Characterisation of molecular mechanisms involved in nevirapine-induced hypersensitivity

Thesis submitted in accordance with the requirements of

the University of Liverpool for the degree of

Doctor in Philosophy

by

Elena Maria Cornejo Castro

March 2014

Declaration

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification.

Elena Maria Cornejo Castro

This research was carried out in the Department of Molecular and Clinical Pharmacology, in the Institute of Translational Medicine, at the University of Liverpool.

Acknowledgements

To everyone who has contributed to this thesis:

Thank you, Danke, Zikomo, Cheers, Terima kasih, Hvala, Gracias, Tapadh leibh,

A'inya, Xiè xie, Grazie, Diolch, Obrigada, Kob khun ka,

Go raibh maith agaibh, Merci, Shukria, Do jeh,

Ta'

Contents

Publications and communications

Published papers

Carr D.F., Chaponda M., Jorgensen A.L., **Castro E.C.**, Van Oosterhout J.J., Khoo S.H., Lalloo D.G., Heyderman R.S., Alfirevic, A., Pirmohamed, M. (2013). ASSOCIATION OF HUMAN LEUKOCYTE ANTIGEN ALLELES AND NEVIRAPINE HYPERSENSITIVITY IN A MALAWIAN HIV-INFECTED POPULATION. Clin Infect Dis, 56, 1330-9.

Manuscripts in preparation

Cornejo Castro E.M., Carr D.F., Jorgensen A.L., Alfirevic A., Pirmohamed M. (2014). HLA-ALLELOTYPE ASSOCIATION WITH NEVIRAPINE-INDUCED HYPERSENSITIVITY: A SYSTEMIC REVIEW OF THE LITERATURE AND META-ANALYSIS.

Cornejo Castro E.M., Carr D.F., Chaponda M., Jorgensen A.L., Rainbow L., Lane B., Ebrahimi B., Khoo S., Lalloo D.G., Alfirevic A., Cossins A.R., Pirmohamed M. (2014). TRANSCRIPTOMIC ANALYSIS INDENTIFIES DIFFERENTIAL REGULATION OF NEUTROPHIL ANTIGEN CD177 MRNA BUT NOT PROTEIN, IN NEVIRAPINE-TREATED HYPERSENSITIVE AND TOLERANT HIV-1 PATIENTS FROM MALAWI.

Carr D.F., Bourgeois S., Chaponda M., **Cornejo Castro E.M**., Alfirevic, A., Khoo S.H., Lalloo D.G., Heyderman R.S., Dandara C., Kampira E., Van Oosterhout J.J., Ssali F., Walker A.S., Novelli G., Borgiani P., Nelson M.R., Holden A., Deloukas P., Pirmohamed M. (2014). GENOME-WIDE ASSOCIATION STUDY OF NEVIRAPINE HYPERSENSITIVITY IN A MALAWIAN HIV-INFECTED POPULATION.

Carr D.F., Chaponda M., **Cornejo Castro E.M.**, Jorgensen A.L., Khoo S.H., Van Oosterhout J.J., Lalloo D.G., Heyderman R.S., Pirmohamed M. (2014). CYP2B6*18 IS ASSOCAITED WITH NEVIRAPINE HYPERSENSITIVITY INDEPENDENTLY OF HLA-C*04:01 IN A MALAWIAN HIV POPULATION. (submitted to the Journal of Antimicrobial Chemotherapy)

Poster presentations

Cornejo Castro E.M., Carr D.F., Marshall V.A., Chaponda M., Lane S., Alfirevic A., Whitby D., Carrington M., Pirmohamed M.: REGULATION OF HUMAN LEUKOCYTE ANTIGEN EXPRESSION AND NEVIRAPINE-INDUCED ADVERSE DRUG REACTIONS IN A MALAWIAN HIV-POSITIVE POPULATION. EACPT, 28 - 31 August, 2013, Geneva, Switzerland

Cornejo Castro E.M., Carr D.F., Marshall V.A., Chaponda M., Lane S., Alfirevic A., Whitby D., Carrington M., Pirmohamed M.: REGULATION OF HUMAN LEUKOCYTE ANTIGEN EXPRESSION AND NEVIRAPINE-INDUCED ADVERSE DRUG REACTIONS IN A MALAWIAN HIV-POSITIVE POPULATION. CESAR Annual Meeting, 27 - 29 June, 2013, Tuebingen, Germany

Cornejo Castro E.M., Carr D.F., Chaponda M., Rainbow L., Lane B., Jorgensen A.L., Alfirevic A., Pirmohamed M.: INVESTIGATION OF HYPERSENSITIVITY REACTIONS TO NEVIRAPINE IN A MALAWIAN COHORT. Drug Hypersensitivity Meeting 5, 11 - 14 April, 2012, Munich, Germany

Abstract

The non-nucleoside reverse transcriptase inhibitor nevirapine (NVP) is widely used in the treatment of human immunodeficiency virus infection in many sub-Saharan countries. However, NVP-treated individuals have a 5% risk of developing hypersensitivity reactions (HSRs), ranging from maculopapular exanthema to life-threatening severe cutaneous eruptions and hepatotoxicity. A number of clinical (gender and CD4+ T cell count) as well as immunogenetic factors (MHC class I and class II alleles) have been associated with NVP hypersensitivity, but the predictive value is poor.

Gene expression analyses provide an unbiased method for determining which immune-related mechanisms are involved in the pathogenesis of NVP hypersensitivity. mRNA microarray analysis demonstrated that expression of *CD177*, a neutrophil specific antigen, was significantly increased in our cohort of NVP-treated patients from Malawi ($p \leq 0.001$), but also after *in vitro* treatment of NVP-hypersensitive patients from Liverpool ($p < 0.05$). Interestingly, CD177 protein expression did not increase in patient samples treated with NVP *in vitro*. A case-control study (n = 288) of NVP-hypersensitive and tolerant patients showed that none of the investigated *CD177* polymorphisms were associated with NVP-induced HSRs. Of the clinical factors analysed, only CD4+ T cell count was significantly associated with NVP hypersensitivity ($p < 0.001$). The polymorphisms rs45441892 and rs10425385 in *CD177* have previously been associated with an increased proportion of CD177-positive granulocytes. We confirmed these results in our NVP-naïve, healthy volunteer cohort (n = 35) for rs10425385, but not rs45441892.

Previous reports have identified *HLA-C*04:01* as a susceptibility marker for NVP-induced HSRs in patients from Malawi. Expression levels of miR-148a, a microRNA known to downregulate HLA-C expression, were analysed in serum samples from NVP-hypersensitive and tolerant individuals (n = 96). However, paradoxically, a statistically significant increase in miR-148a expression was found in NVP-hypersensitive patients at the time of reaction ($p = 0.008$).

Additionally, the serum expression levels of 84 miRNAs were analysed in hypersensitive and tolerant patients treated with NVP (n = 24). Twenty-one miRNAs were differentially expressed in tolerant and hypersensitive samples. Of these miRNAs, miR-205 showed the highest increase in NVP-hypersensitive patients ($p < 0.01$).

Besides *HLA-C*04*, several other HLA-alleles have been reported as risk factors for NVP hypersensitivity. A summary meta-analysis of published data indicated that four HLA-allelotypes (*HLA-B*35, -B*58:01, -C*04, -DRB1*01*) might be common risk factors of NVP-induced HSRs for different ethnicities.

The results presented in this thesis highlight that various genetic, immunological and clinical factors may contribute to the pathogenesis of NVP hypersensitivity. Further understanding of the complex interactions may ultimately lead to the characterisation of true causative associations facilitating the precise prediction of hypersensitivity reactions to NVP.

Abbreviations

Acronym	
12-OH-NVP	12-hydroxy nevirapine
2-OH-NVP	2-hydroxy nevirapine
3-OH-NVP	3-hydroxy nevirapine
4-COOH-NVP	4-carboxyl acid nevirapine
8-OH-NVP	8-hydroxy nevirapine
ABC	Abacavir
ABC	ATP-binding cassette transporter
ADME	Absorption, distribution, metabolism, excretion
ADR	Adverse drug reaction
AIDS	Acquired immune deficiency syndrome
ALT	Alanine aminotransferases
ANCA	Anti-neutrophil cytoplasmatic antibody
ANOVA	analysis of variance
APC	Antigen presenting cell
ART	Antiretroviral therapy
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
bp	Base pair
cADR	Cutaneous adverse drug reaction
CBZ	Carbamazepine
cDNA	Complementary DNA
CI	Confidence interval
CSF3	Colony stimulating factor 3
CTL	Cytotoxic T cell
CYP	Cytochrome P450 enzyme
DAMPs	Damage-associated molecular patterns
DEPC	Diethylpyrocarbonate
DIHS	Drug-induced hypersensitivity syndrome
DILI	Drug-induced liver injury
DME	Drug metabolising enzyme
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DoTS	Dose relatedness, timing, patient susceptibility
DRESS	Drug rash with eosinophilia and systemic symptoms
EDTA	Ethylendiaminetetraacetic acid
EFV	Efavirenz
ELISpot	Enzyme-linked immunospot
EMA	European Medicines Agency
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FAM	6-carboxyfluorescein
FDA	U.S. Food and Drug Administration

FDR	False discovery rate
FITC	fluorescein isothiocyanate
fMLP	N-formyl-methionyl-leucyl-phenylalanine
G-CSF	Granulocyte-colony stimulating factor
GWAS	Genome wide association study
HAART	Highly active antiretroviral therapy
HBSS	Hank's Balanced Salt Solution
HHV	Human herpes virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMGB1	high mobility box protein 1
HNA-2a	Human neutrophil antigen 2a
HSR	Hypersensitivity reaction
HSS	Hypersensitivity syndrome
HWE	Hardy-Weinberg equilibrium
ICAM-1	Intracellular adhesion molecule-1
ICS	Intracellular cytokine staining
IL	Interleukin
IPD	Individual patient data
IQR	Interquartile range
JAK-STAT	Janus kinase-signal transducers and activators of transcription
JAK2	Janus kinase 2
LD	Linkage disequilibrium
LTR	Long terminal repeat
M	Molar
MAF	Minor allele frequency
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
mg	Milli grams
MHC	Major histocompatibility complex
miRNA	MicroRNA
ml	Milli litre
mM	Milli molar
MPE	Maculopapular exanthema
mRNA	Messenger RNA
MTCT	Mother-to-child transmission
NAT	N-acetyltransferase
NF-κB	Nuclear factor-κB
NFQ	non-fluoerescence quencher
NIR	Nevirapine-induced rash
NNRTI	Non-nucleoside and nucleotide reverse transcriptase inhibitor
NRTI	Nucleoside and nucleotide reverse transcriptase inhibitor
NSAID	Non-steroidal anti-inflammatory
NTC	No template control
NVP	Nevirapine
OR	Odds ratio
ORF	Open reading frame

PBMCs	peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PECAM-1	Platelet endothelial adhesion molecule-1
PGLYRP1	Peptidoglycan recognition protein 1
PI	Protease inhibitor
PR3	Proteinase 3
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PRV-1	Polycythemia rubra vera 1
qPCR	Real-time polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RT	Reverse transcriptase
SHIP2	SH_2-containing phosphoinositide 5'-phosphatase 2
SJS	Stevens Johnson syndrome
SLC	Solute carrier family
SNP	Single-nucleotide polymorphism
TCR	T cell receptor
TEN	Toxic epidermal necrolysis
U	Activity unit
UGT	Uridine diphosphate gluguronosyltransferase
UTR	Untranslated region
WHO	World Health Organisation
µM	Micro molar

Chapter 1

General Introduction

Contents

Figures

Tables

1.1 Drug-induced hypersensitivity reactions

In the UK, drug-related adverse effects account for 6.5% of hospital admissions (PIRMOHAMED *et al.*, 2004), while in the US adverse drug reactions (ADRs) represent the sixth leading cause of death (LAZAROU *et al.*, 1998). It has been estimated that genetic variations can contribute 20-50% to the interindividual variability in drug response and safety. Pharmacogenetics and pharmacogenomics aim to optimise drug therapy based on each individual's genetic profile and thus decrease the likelihood of adverse effects (EVANS and MCLEOD, 2003).

1.1.1 Definitions of adverse drug reactions

The World Health Organization (WHO) first defined an ADR as "a response to a drug that is noxious and unintended and occurs at doses normally used in man for the prophylaxis, diagnosis or therapy of disease, or for modification of physiological function" (WHO, 1972).

In 2000, Edwards and Aronson suggested a more extensive definition: "An appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product" (EDWARDS and ARONSON, 2000).

The European Parliament and Council of the European Union proposed in 2010 to amend the definition of ADRs "to ensure that it covers noxious and unintended effects resulting not only from the authorised use of a medicinal product at normal doses, but also from medication errors and uses outside the terms of the marketing authorisation, including the misuse and abuse of the medicinal product" (EU, 2010).

Though all definitions mentioned above are suitable for describing an ADR, the latter two address more specifically serious, life-threatening conditions along with reactions caused by prescription errors and off-label use.

1.1.2 Classification of adverse drug reactions

Adverse drug reactions were first divided into dose-dependent (type A) and non-dose-dependent (type B) reactions (RAWLINGS and THOMPSON, 1977):

Type A reactions represent the most common type of ADRs. They are based on the known pharmacological function of the drug. Although their severity can range from mild to serious reactions, they are characterised by a low morbidity and mortality rate (EDWARDS and ARONSON, 2000; PIRMOHAMED *et al.*, 2004; UETRECHT, 2007). Age, drug-drug interactions, variations in pharmacokinetic and pharmacodynamic parameters as well as environmental factors may increase the chance of developing this type of ADR (MEYER, 2000). Examples of type A reactions include an increase in the bleeding risk with warfarin as well as hypotension caused by antihypertensives.

Type B reactions affect only a small proportion of patients and are unpredictable, as they do not relate to the pharmacological function of the drug. They generally comprise more severe reactions and are a leading cause of drug withdrawal during drug-development. Type B reactions are often also known as idiosyncratic drug reactions (PHILLIPS and MALLAL, 2010; PIRMOHAMED *et al.*, 1998; UETRECHT and NAISBITT, 2013). They will be further discussed in chapter 1.1.3.

Later, four additional reaction types (type C-F) were proposed to account for ADRs not covered by the categories mentioned above (EDWARDS and ARONSON, 2000). In addition, Aronson *et al*. suggested a classification system based upon Dose dependency, Timing and individual Susceptibility [DoTS (ARONSON and FERNER, 2003)].

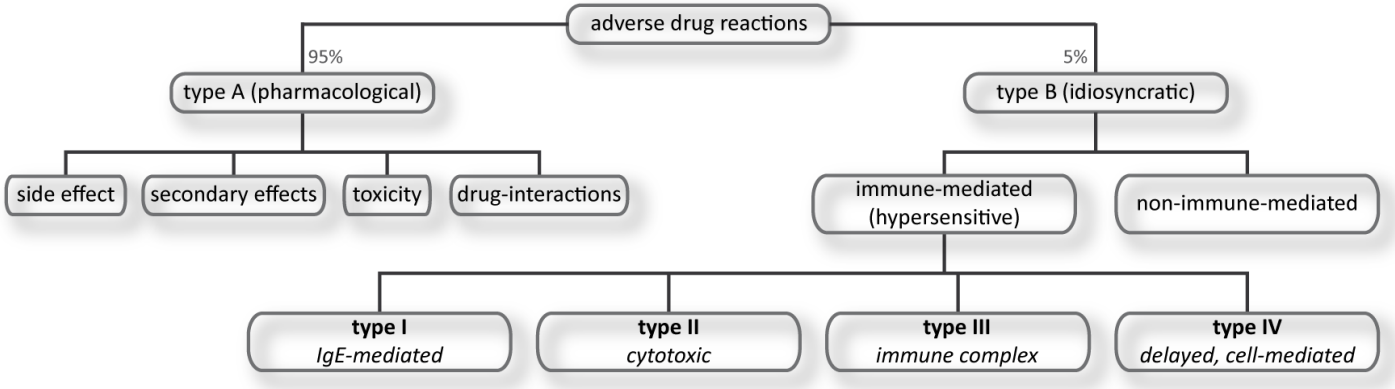
1.1.3 Characteristics of idiosyncratic adverse drug reactions

Type B or idiosyncratic adverse drug reactions only occur in susceptible individuals. They are characterised by a delayed onset of symptoms varying from a few minutes to multiple hours or days after drug exposure. Further,

adverse drug effects manifest quicker if patients, who have previously been exposed to a specific drug, are rechallenged (PIRMOHAMED, 2010; UETRECHT and NAISBITT, 2013). Another characteristic is that these reactions do not show a simple dose-response relationship, i.e. the risk does not increase proportionally with an increase in dose (UETRECHT, 2007). Idiosyncratic reactions can be further subdivided into immune-mediated and non-immune-mediated reactions. The terms immune-mediated and hypersensitive are often used interchangeably, and in this thesis the term hypersensitivity reactions (HSRs) will be used throughout.

Gell and Coombs first classified HSRs into four groups based on the underlying immune mechanism (GELL and COOMBS, 1968). This classification system is summarised in figure 1.1 and includes antibody mediated reactions type I – type III as well as the T cell mediated type IV. More recently, type IV reactions have been further subdivided based on the effector cells and mediators involved in the immune response [type IVa – IVd (PICHLER, 2003)]. However, this classification represents a very simplified representation of the complex developments occurring within the immune system and often multiple reaction types can be observed simultaneously (PICHLER *et al.*, 2010).

Several demographic and clinical factors, such as age, gender, concomitant diseases, co-medication and genetic polymorphisms are known to predispose to ADRs (BECQUEMONT, 2009).



Clinical manifestation	Urticaria, anaphylaxis	Neutropenia, hemolytic anemia	Vasculitis, rash, fever	Macupapular rash, allergic contact dermatitis
Time to reaction after drug exposure	Minutes to 1 hour	Hours to a day	3 - 10 hours	48 - 72 hours
Cell type	B cells	B cells	B cells	T cells
Drug binding	Presumed covalent	Presumed covalent	Presumed covalent	Covalent and noncovalent
Type of immune response	Drug-IgE complex, relase of inflammatory mediators	Specific IgG or IgM antibodies and Fc receptor interaction	Drug-IgG complexes and complement activation or Fc receptor interaction	MHC presentation to T cells *IVa* T helper 1 *IVb* T helper 2 *IVc* cytotoxic T cell *IVd* T cells
Pathological mechanism	Mast-cell degranulation	Fc receptor-dependent cell destruction	Immunocomplex deposition	*IVa* monocyte activation *IVb* eosinophilic inflammation *IVc* CD4 or CD8 T cell mediated killing *IVd* neutrophil recruitment and activation

Fig. 1.1: Classification of immune-mediated adverse drug reactions

Revised classification of adverse drug reactions with extended Gell and Coombs classification of idiosyncratic reactions (Adapted from: PICHLER, 2003; PIRMOHAMED *et al.*, 2004; THIEN, 2006). *Ig* immunoglobin, *MHC* major histocompatibility complex

1.1.4 Clinical features of idiosyncratic drug reactions

Idiosyncratic drug reactions can affect almost any organ system in the human body. Each drug is associated with its own specific range of reactions, affecting single organs or multiple organ systems. However, the clinical manifestations caused by a drug may vary between patients (PHILLIPS and MALLAL, 2010; UETRECHT and NAISBITT, 2013).

Drug hypersensitivity in the skin

Skin reactions are the most common type of hypersensitivity reactions, with maculopapular exanthema (MPE) accounting for 90% of cutaneous ADRs [cADRs (BIGBY, 2001; HUNZIKER *et al.*, 1997)]. These reactions appear first as isolated patches and lesions, which then spread in a symmetrical fashion to the extremities. They appear 1–2 weeks after first drug administration and symptoms usually resolve following drug withdrawal (ROUJEAU, 2005).

A more severe reaction of drug-induced hypersensitivity, which involves multiple organs and white blood cell abnormalities, is drug reaction with eosinophilia and systemic symptoms (DRESS). Other terms commonly used to refer to the same syndrome are drug-induced hypersensitivity syndrome (DIHS) and hypersensitivity syndrome (HSS). DRESS is often characterised by an acute onset of rash, fever as well as hematologic defects and may include one or more of the following symptoms: lymphadenopathy, carditis, pneumonitis, hepatitis and nephritis (ROUJEAU, 2005; UETRECHT and NAISBITT, 2013). Hepatitis is the most common organ manifestation, with liver failure being the prevalent cause of mortality [mortality rate approximately 10% (HAUSMANN *et al.*, 2010; PHILLIPS and MALLAL, 2010)]. These reactions begin 2-6 weeks after treatment initiation. Reactivation of herpes virus infections (such as human herpes virus 6 (HHV-6) and HHV-7, cytomegalovirus and Epstein-Barr virus) has been associated with DRESS (DESCAMPS *et al.*, 1997; KOMURA *et al.*, 2005; SEISHIMA *et al.*, 2006; SUZUKI *et al.*, 1998). This association is not completely understood; Picard *et al.* proposed that virus reactivation may act as a pathogenic mechanism of DRESS in which drug administration triggers virus reactivation and subsequently leads to the

activation of virus-specific T cells, which wrongly attack non-specific tissues (PICARD *et al.*, 2010). Drugs most commonly associated with DRESS are anticonvulsants (e.g. carbamazepine (CBZ)), sulfonamides, allopurinol and antiretrovirals (e.g. abacavir (ABC) and nevirapine (NVP)).

The most severe cADRs are Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). They are now considered to be different severity variants of the same disease, distinguished by the extent of skin detachment: SJS involves less than 10% of the body surface area, TEN more than 30% and the SJS/TEN overlapping syndrome affects 10-30%. The symptoms emerge 1-3 weeks after first drug treatment and normally begin with a sudden onset of fever and malaise, followed by a painful rash and the formation of blisters. At least two mucous membranes are usually involved and ocular symptoms develop occasionally. Histologically, SJS and TEN are characterised by extensive keratinocyte apoptosis (HAUSMANN *et al.*, 2010; PEREIRA *et al.*, 2007; PHILLIPS and MALLAL, 2010). This leads to separation of the dermis and epidermis, with the degree of necrosis correlating to the amount of mononuclear infiltrate in the dermis [mostly CD8+ T cells (HASHIZUME *et al.*, 2002)]. The mortality rate reaches 10% in SJS and nearly 30% in TEN patients, with sepsis and pulmonary manifestations being the major cause of death (ROUJEAU, 2005; SVENSSON *et al.*, 2001). Although these reactions only occur in about two patients per million each year, the incidence of SJS and TEN has been reported to be higher in human immunodeficiency virus (HIV) infected patients (MITTMANN *et al.*, 2012; RZANY *et al.*, 1993). At least 70% of all cases are classified as drug-induced reactions; the drugs associated with a higher risk of developing SJS/TEN are anticonvulsants (CBZ, phenytoin, phenobarbital), allopurinol and NVP (PEREIRA *et al.*, 2007; ROUJEAU, 2005).

Drug-induced liver injury

Idiosyncratic drug-induced liver injury (DILI) occurs at therapeutic doses with no clear evidence of dose-dependency. The onset of DILI is variable, with reactions appearing one week to several months after first drug administration. It is further characterised by adaptation, in which mild liver injury determined

by elevated alanine transaminase (ALT) and aspartate aminotransferase (AST) levels, resolves in most patients despite treatment continuation (UETRECHT, 2007). Although DILI is classified as rare, occurring from 1 in 1000 to 1 in 100.000 patients, DILI is the most frequent cause for the discontinuation during drug-development and withdrawal of already established pharmaceuticals (CHALASANI and BJORNSSON, 2010; KAPLOWITZ, 2001; LEE, 2003). Drugs or their reactive metabolites have been shown to damage hepatocytes through various cellular mechanisms either by direct interaction with mitochondrial molecules, indirect increase of intracellular stress (e.g. promoting oxidative or cytoskeletal stress as well as DNA damage) or activation of the immune system through haptenization [see chapter 1.2 (KAPLOWITZ, 2004)]. Hepatocellular damage in DILI accounts for the majority of cases of acute liver injury in the US, which requires liver transplantation or results in the death of patients (KAPLOWITZ, 2005). If elevated ALT levels are accompanied by both an increase in bilirubin and the appearance of jaundice, the mortality rate increases to 10-50% (ZIMMERMAN, 1999).

Recent data identified many human leukocyte antigen (HLA) alleles as genetic markers of drug-induced HSRs; the nature of these associations will be discussed in section 1.3 in more detail.

1.2 Mechanistic hypotheses of hypersensitivity reactions

Although the mechanisms facilitating the recognition of conventional antigens by T cell receptors (TCRs) have been explored in detail, the mechanisms by which immune-mediated ADRs elicit an immune reaction are still not clear. In general, peptides are presented on antigen-presenting cells (APCs) through major histocompatibility complex (MHC) molecules to TCRs. Thus the MHC/TCR interaction promotes an antigen-specific immune response.

A number of pathways that suggest how small molecules such as drugs are able to interact and activate the immune system are outlined below. These mechanisms seem to complement each other and not to occur separately.

1.2.1 The major histocompatibility complex

Originally identified in mice, HLA genes in humans encode transmembrane proteins that display endogenous and pathogen-derived peptides on the cell surface triggering a T cell mediated immune response. Located on the short arm of chromosome 6 at 6p21.3, the MHC complex contains more than 200 genes, many of which possess a function in innate and adaptive immunity. Combinations of HLA-genes feature a high level of linkage disequilibrium (LD), leading to alleles in adjacent loci to be inherited as haplotypes (STENZEL *et al.*, 2004).

MHC classification and structure

The polygenic MHC molecules can be categorised into MHC class I and MHC class II molecules. All MHC genes are co-dominantly expressed on the cell surface. The three classical MHC I loci, *HLA-A*, *-B* and *-C*, as well as the three classical MHC II loci, *HLA-DR*, *-DQ* and *-DP* belong to the most highly polymorphic genes present in the human genome (MURPHY *et al.*, 2012).

The class I genes code for the polymorphic α heavy chain consisting of three extracellular domains (α1, α2 and α3), which is expressed in non-covalent association with the invariant light chain β2-microglobulin (β_2m), encoded on chromosome 15. The peptide-binding groove is composed of the α1 and α2 subdomains. In addition to the peptide binding domain and the immunoglobulin like domain (α3), MHC class I molecules possess a transmembrane region as well as a cytoplasmatic tail. HLA class I receptors are expressed on almost every nucleated cell, although expression levels may vary between alleles.

By contrast, MHC class II molecules are expressed by APCs such as dendritic cells, B cells and macrophages only. The HLA class II genes code for two

polymorphic chains α and β, that contain a peptide-binding domain (α1 or β1), an immunoglobulin like domain (α2 or β2) as well as a transmembrane region and a cytoplasmatic tail [figure 1.2 A (BHARADWAJ *et al.*, 2012; KLEIN and SATO, 2000)].

HLA-alleles can differ by 1 to 30 amino acid residues and these polymorphisms result mainly in amino acid changes involved in peptide recognition and binding. The nomenclature of HLA-alleles was initially based on serological typing. However, the use of genotyping methods led to an increase in the number of known alleles and the introduction of an eight-digit system, which allows HLA-alleles to be differentiated [figure 1.2 B (MARSH *et al.*, 2010; TURNER, 2004)]. MHC class I molecules that bind to identical peptides have been organised in supertypes (SETTE and SIDNEY, 1998).

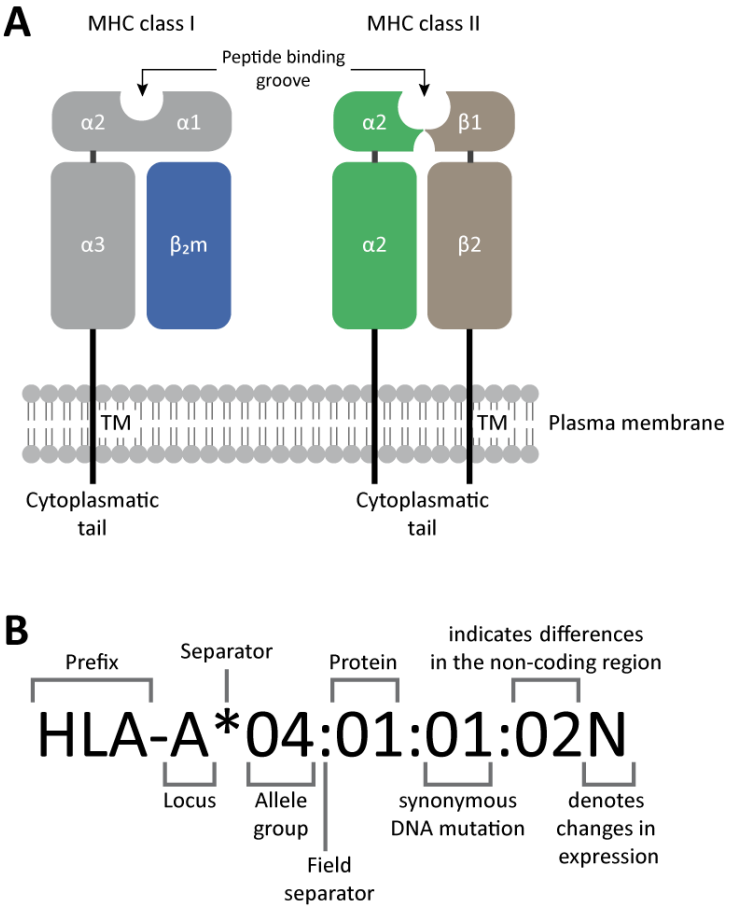


Fig. 1.2: Structure and nomenclature of HLA class I and class II molecules
(**A**) MHC class I: α chain, consisting of two peptide-binding domains (α1 and α2) and an immunoglobulin like domain (α3); the beta2-microglubulin (β2m) is the light chain of the molecule. MHC Class II: α and β chains have a peptide-binding domain (α1 or β1) as well as an immunoglobulin like domain (α2 or β2). Both molecules feature a transmembrane domain (TM) and a cytoplasmatic tail (adapted from: KLEIN and SATO, 2000). (**B**) Nomenclature of HLA-alleles (adapted from: ROBINSON *et al.*, 2011).

Antigen presentation and T cell interaction

Cytosolic proteins are degraded by the proteasomes and the emerging peptides are either processed into amino acids directly in the cytosol or transferred into the endoplasmic reticulum (ER). Within the ER, peptides bind to MHC class I molecules and the MHC/peptide complex is then transported via vesicles to the cell surface. The antigen presentation by MHC class II requires exogenous proteins to be endocytosed. Vesicles carrying fully assembled class II molecules merge with endosomes forming the MHC class II compartment where peptides derived from exogenous proteins are loaded into the binding groove. The loaded class II molecule is then transported to the cell surface (KLEIN and SATO, 2000; MURPHY *et al.*, 2012). Although the MHC class I and class II pathways have been described to bind exclusively to intracellular or extracellular proteins, it has been shown that cross-presentation allows class I molecules to be loaded with exogenous peptides internalised through phagocytosis (JOFFRE *et al.*, 2012). At the same time, fusion of autophagosomes with MHC class II compartments leads to the loading of intracellular antigens onto MHC class II molecules (MUNZ, 2012).

The closed conformation of the MHC class I peptide binding groove accommodates shorter peptides than the open-ended class II groove (8–9 amino acids versus 12-25). The specificity of antigens capable of binding to a particular MHC allele is defined by a small number of amino acid residues that interact with so-called pockets in the peptide-binding groove.

The cell surface is the site of the interaction between the MHC/peptide complex and the TCR. Mature T cells are differentiated by the expression of either CD4 or CD8 co-receptors, resulting in CD4+ and CD8+ T cell subtypes, which interact with MHC class II and MHC class I molecules respectively. While the TCR binds to the groove and the peptide directly, CD4 and CD8 co-receptors engage MHC molecules outside the peptide-binding groove. The interaction of the TCR with the MHC/peptide complex stimulates the antigen-specific proliferation and differentiation of the T cell. CD8+ T cells differentiate into cytotoxic T cells, whereas CD4+ T cells differentiate into T helper cells (ALBERTS *et al.*, 2008; BHARADWAJ *et al.*, 2012; KLEIN and SATO, 2000).

1.2.2 Hapten hypothesis

Based on the observations from Landsteiner *et al.*, the hapten hypothesis explains how low-molecular weight compounds that are not able to initiate T cell activation, become antigenic after covalent binding to proteins (LANDSTEINER and JACOBS, 1935; UETRECHT, 2008). Drug-protein conjugates, also known as haptens, are small chemically reactive molecules that are able to bind covalently to any kind of endogenous protein or peptide (intracellular, membrane bound or soluble). Theoretically, the type, location and amount of modified peptides may activate distinct immunological pathways leading to the diverse clinical manifestations of HSRs. Most drugs do not exhibit chemical reactive qualities, but form reactive products upon metabolism. This is called the pro-hapten concept (ADAM *et al.*, 2011; LI and UETRECHT, 2010; PICHLER *et al.*, 2006).

For the initiation of a T cell dependent immune response, small molecules or drugs as well as their reactive metabolites must bind covalently to endogenous proteins, forming drug-protein adducts. These adducts are endocytosed and processed by APCs which then display the drug-modified peptides through MHC molecules. Once the peptide/MHC complex is recognised by the TCR, naïve T helper cells differentiate into T_H1 and T_H2 effector cells. The recognition of these modified adducts by TCRs is referred to as signal 1 [figure 1.3 A (PICHLER *et al.*, 2006; PIRMOHAMED *et al.*, 2002; UETRECHT, 2007)]. Effector cells induce a phagocyte-dependent inflammation by activating cytotoxic T cells and macrophages but can also stimulate B cell dependent antibody generation and secretion (ALBERTS *et al.*, 2008).

1.2.2 Danger hypothesis

Drug haptenation occurs frequently after bioactivation, yet only a small number of patients are susceptible for drug-induced HSRs. This specificity is further addressed by the danger hypothesis.

As the first immunological hypothesis to address the specificity of the immune system, the self-nonself model by Burnet *et al.* describes how naturally

generated peptides are tolerated by the immune system and only foreign proteins generate an immune response (BURNET, 1961). Matzinger *et al.* questioned this concept; she proposed that in the absence of an adjuvant, nonself proteins could not generate an immune response (MATZINGER, 1994). Prior to the interaction of the MHC/TCR complex, APCs have to be activated by a so-called danger signal (figure 1.3 B). This signal is generated through cell damage and includes a range of different molecules like cytokines, heat shock proteins and annexins. The activation of APCs leads to the upregulation of co-stimulatory molecules, mainly B7. The interaction between B7 and CD28 on the T cell membrane is referred to as signal 2 (LI and UETRECHT, 2010; UETRECHT, 2007). Without the co-stimulatory signal, the MHC/TCR interaction alone results in immune tolerance.

In drug-induced HSRs cell damage induced by the formation of reactive metabolites and drug-protein conjugates may represent the danger signal. Simultaneously, these molecules can act as antigens in MHC/TCR signalling, providing the required signals to initiate an immune response (PIRMOHAMED *et al.*, 2002). Other factors, such as different kinds of tissue injury, inflammation and concomitant viral infections, have been shown to increase the risk of HSRs (PIRMOHAMED, 2007; PIRMOHAMED *et al.*, 2002).

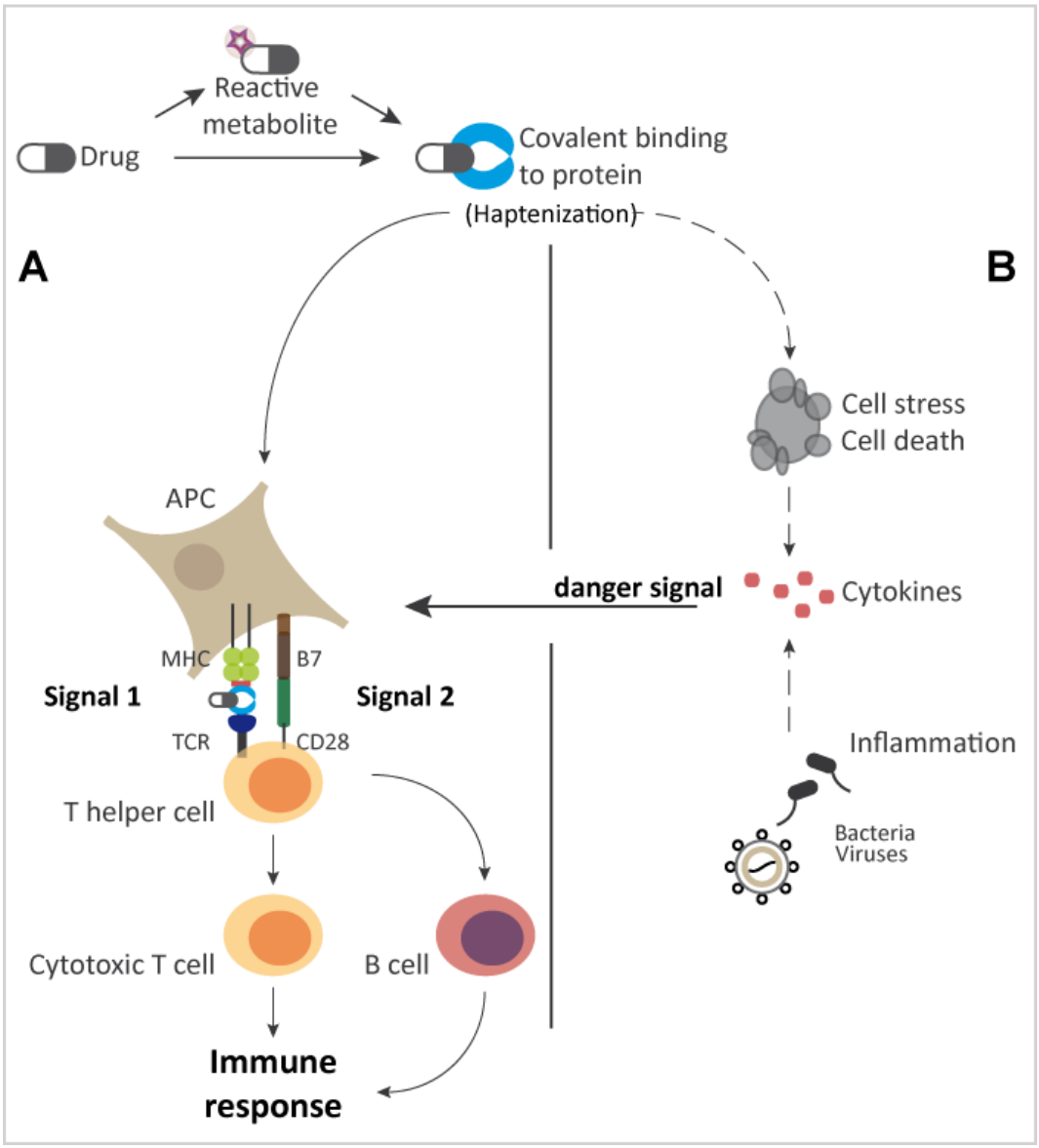


Fig. 1.3: The hapten and danger hypotheses

(**A**) *Hapten hypothesis.* Covalent binding of drugs or their reactive metabolites to endogenous proteins leads to the formation of drug-protein conjugates, also known as haptens. These are processed by APCs leading to the MHC presentation of the antigen. The recognition of the haptenated peptides by TCRs (signal 1) promotes cell-activation and differentiation of effector cells that induce a cell dependent or antigen-mediated immune response. (**B**) *Danger hypothesis:* In addition to signal 1, the co-stimulation of T helper cells through the interaction of CD28 with the B7 molecule on the APC (signal 2) is required for an immune reaction. Drugs, inflammation or infection lead to cell damage and cytokine release (danger signal), which activate APCs and leads to the upregulation of the co-stimulatory molecule B7 (Adapted from: KAPLOWITZ, 2005). *APC* antigen presenting cell, *MHC* major histocompatibility complex, *TCR* T cell receptor

1.2.3 Pharmacological interaction (p-i) concept

In 2002, Pichler *et al.* first suggested that drugs elicit an immune reaction by binding reversibly to certain MHC or TCR molecules [figure 1.4 (PICHLER, 2002)]. This hypothesis is known as 'pharmacological interaction of drugs with immune receptors' or p-i concept and is facilitated by the formation of Van der Waals and hydrogen bonds. Whether drug interaction occurs first with the MHC and is followed by the interaction of the drug-MHC complex to the TCR or whether TCRs represent the primary target for drug binding, has not been demonstrated. Pichler *et al.* proposed that a drug-specific activation of cross-reactive memory T helper cells allows a much faster activation of a secondary immune response, leading to an enhanced cell- or antibody-mediated immune reaction (ADAM *et al.*, 2011; PICHLER *et al.*, 2006). Like the hapten hypothesis, the p-i concept does not discuss the subject of signal 2 (UETRECHT, 2007).

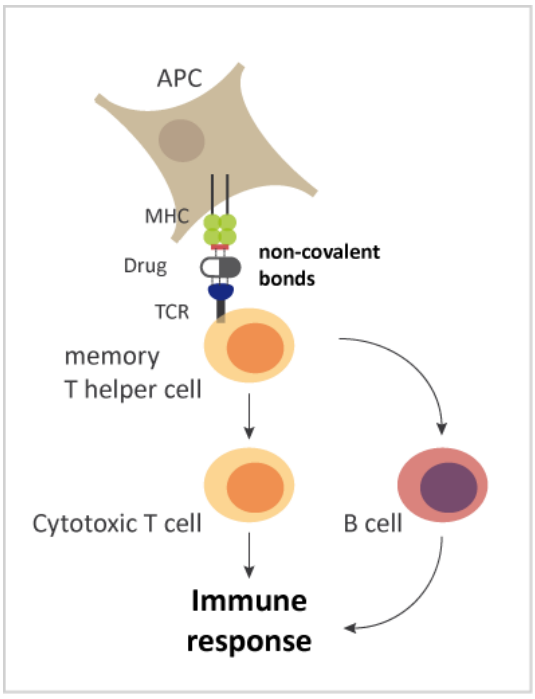


Fig. 1.4: Pharmacological interaction (p-i) hypothesis
Chemical inert drugs reversibly bind to MHC or TCR molecules. The interaction between the drug-MHC complex and the TCR results in the activation of a secondary immune response, which results in a faster proliferation and expansion of memory T helper cells and the subsequent initiation of cell- or antibody-mediated immune pathways. *APC* antigen presenting cell, *MHC* major histocompatibility complex, *TCR* T cell receptor

1.2.4 Altered HLA-peptide repertoire hypothesis

Recently, a new mechanism for ABC-induced hypersensitivity has been identified [for more details see chapter 1.3.1 (ILLING *et al.*, 2012; OSTROV *et al.*, 2012)]. Abacavir hypersensitivity is associated with *HLA-B*57:01* and T cell clones from *B*57:01* positive individuals were shown to proliferate in an ABC dependent manner (CHESSMAN *et al.*, 2008; HETHERINGTON *et al.*, 2001). Illing *et al.* were able to demonstrate that ABC binds non-covalently to the HLA-B*57:01 binding grove and that this interaction consequently promotes the binding of novel peptides in the presence of the drug.

Thus, the specific but reversible interaction of drugs with MHC class I molecules facilitates the binding of novel self-peptides and simultaneously prevents known peptide ligands from binding. This alteration of the peptide repertoire presented by MHC molecules leads to the activation of a distinct subset of TCRs leading to a modified immune response (figure 1.5).

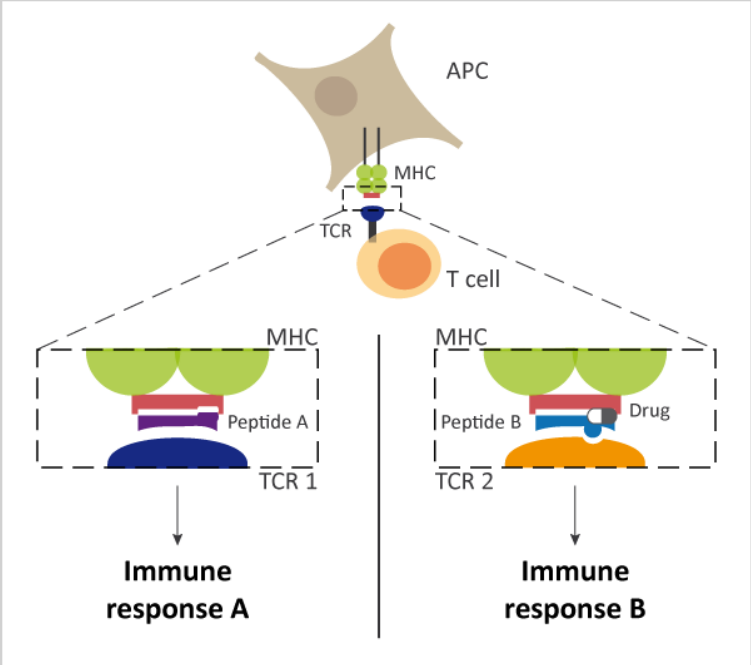


Fig. 1.5: Drug-induced modifications in the peptide repertoire presented by MHC molecules.
The binding of novel peptides (peptide B) in the presence of the drug leads to the activation of distinct TCRs, which triggers an immune response (Adapted from: OSTROV *et al.*, 2012). *APC* antigen presenting cell, *TCR* T cell receptor, *MHC* major histocompatibility complex

1.3 Pharmacogenetics of idiosyncratic drug reactions

Genetic variations that contribute to the inter-individual variability in the efficacy and safety of therapeutics are very diverse and involve genes encoding drug-metabolising enzymes (DMEs) and drug transporters (discussed in section 1.3.2) but also immunogenic factors, which have been the main focus of HSRs [see section 1.3.3 (MA and LU, 2011)]. Besides genomic variation, individual differences in drug response may also be based on physiological and environmental factors, such as age, gender, co-existent diseases, smoking and alcohol abuse.

1.3.1 Drug metabolism

The metabolism of xenobiotics is the main elimination pathway of toxins, carcinogens and pharmaceutical molecules. Taken together absorption, distribution, metabolism and excretion (ADME) of compounds determines their bioavailability, and their efficacy and safety. Specialised enzymes, also called DMEs, facilitate the metabolism or biotransformation of substances and are divided into two categories: Phase I and Phase II [summarised in table 1.2 (WILLIAMS, 1959)]. First introduced in 1992, the elimination of detoxification products by drug transporter proteins has been categorised as Phase III metabolism (ISHIKAWA, 1992). The main site of drug metabolism is the liver, but extrahepatic tissues such as the intestine, kidney and lung feature low levels of enzymatic activity. Enzymes and transporters are also found in certain types of white blood cells (PARK *et al.*, 1995).

As will be discussed below, the majority of idiosyncratic drug reactions are mediated by the immune system. Chemically reactive metabolites may form protein adducts capable of interfering with cellular pathways and initiate a novel immune response. Thus, the formation of reactive metabolites as well as the disruption of detoxification processes followed by the formation of covalent bonds to endogenous peptides may trigger a specific immune reaction.

The basic principles of drug metabolism will be described below. Genetic polymorphisms altering the bioactivation of drugs may affect the predisposition of individuals to drug hypersensitivity and will therefore be discussed in chapter 1.3.3.

Phase I reactions

All reactions catalysed by DMEs result in the conversion of lipophilic drugs into more hydrophilic or polar metabolites that are easier to excrete from the body. Phase I reactions include oxidation, reduction and hydrolysis and are also classified as functionalization reactions, which unmask or introduce functional groups. One of the most common reactions is oxidation catalysed by the cytochrome P450 (CYP) enzymes. The CYP enzyme system consists of polypeptides bound to the cytoplasmatic side of the ER. It is particularly expressed in the liver but also in the lung, kidney, skin and heart. Cytochrome P450 molecules are grouped into families and subfamilies based on their sequence homology. The three main families involved in the human drug metabolism are CYP1, CYP2 and CYP3 (NEBERT *et al.*, 1987), with the five major isoforms CYP1A2, 2C9, 2C19, 2D6 and 3A4 being responsible for the metabolism of 90% of drugs (TARANTINO *et al.*, 2009). Additional enzymes conducting Phase I oxidation include alcohol and aldehyde dehydrogenases, Flavin-containing monooxygenases, xanthine oxidases and others (listed in table 1.2).

Depending on their chemical features metabolites that have undergone Phase I metabolism can be either excreted from the body or have to undergo Phase II biotransformation.

Phase II reactions

In Phase II, modified substrates formed in Phase I or xenobiotics that possess functional groups, are conjugated with another molecule. These reactions are catalysed by transferases; the conjugated products have increased molecular weight and their polar properties allow them to be easily excreted from the

body. Glucuronidation is the most common Phase II reaction. This reaction is facilitated by uridine disphospate glucuronosyltransferases (UGTs) that are primarily expressed in the liver, kidney and the intestine. The most important UGTs in man include UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7 and 2B15 (MINERS *et al.*, 2006).

Organ and tissue specific metabolic bioactivation of drugs may play a role in the occurrence of drug hypersensitivity. Besides detoxification, bioactivation of certain Phase I and Phase II enzyme substrates can lead to the formation of reactive metabolites that interact with endogenous polypeptides (PARK *et al.*, 2011; SANDERSON *et al.*, 2006). The formation of reactive metabolites in Phase I is generally catalysed by CYP enzymes, whereas conjugates of acyl glucuronides form electrophilic metabolites in Phase II (OJINGWA *et al.*, 1994). These metabolites are able to form drug-protein adducts that either cause cell damage directly or trigger an antigen specific immune response.

Drug transporters involved in Phase III metabolism

Drug transporters can be divided into the solute carrier (SLC) transporter or adenosine triphosphate (ATP)-binding cassette (ABC) transporter superfamily. The ABC transporter family comprises primarily active transporter proteins that require ATP hydrolysis for the transport of substrates across the membrane. Expressed at the intestine and the liver, these transporters can decrease the oral bioavailability of xenobiotics and their metabolites through rapid biliary excretion (AMACHER, 2012; DIETRICH *et al.*, 2003).

Table 1.1: Phase I and Phase II drug metabolising enzymes

Pathway	Enzyme	Abbreviation	Subcellular location
Phase I - Oxidation			
	Alcohol dehydrogenase	ADH	Cytosol
	Aldehyde dehydrogenase	ALDH	Cytosol, mitochondria
	Aldehyde oxidase	AO	Cytosol
	Aldo-keto reductase	AKR	Cytosol
	Cytochrome P450	CYP	ER
	Diamine oxidase	DAO	Cytosol
	Flavin-containing monooxygenase	FMO	ER
	Monoamine oxidase	MAO	Mitochondria
	Prostagladin H synthase	PGHS	ER
	Xanthine oxidase/ dehydrogenase	XO/ XDH	Cytosol
Phase I - Hydrolysis			
	Carboxylesterase	CE	ER, cytosol, lysosome
	Epoxide hydrolase	EH	ER, cytosol
	β-Glucuronidase		Lysosomes, ER
	Arylesterases/ Paraoxonases	PON	Cytosol
	Pseudocholinesterase/ butyrylcholinesterase	BuChE	Cytosol
	Peptidase		Lysosomes
Phase I - Reduction			
	Azo- and nitro-reductase		ER, cytosol
	Carbonyl reductase		Cytosol, ER
	Disulfide reductase		Cytosol
	Quinone reductase	NADPH	Cytosol, ER
	Sulfoxide reductase		Cytosol
	Reductive dehydrogenase		ER
Phase II – Conjugation			
	Uridine disphospate glucuronyltransferase	UGT	ER
	Sulfotransferase	SULT	Cytosol
	Gluthatione S-transferase	GST	Cytosol, ER
	Amino acid conjugate system		Mitochondria, ER
	N-Acetyltransferase	NAT	Cytosol
	Methyltransferase		Cytosol, ER

(Adapted from: KHOJASTEH, 2011)

1.3.2 Genetic polymorphisms of drug transporters and metabolizing enzymes

To date, many polymorphisms affecting pharmacokinetic (drug concentration) as well as pharmacodynamic (drug action) parameters have been discovered.

These gene polymorphisms include gene copy number variations, such as deletions or amplifications of DNA sections, as well as single-nucleotide polymorphisms (SNPs). Gene polymorphism of either Phase I or Phase II enzymes can lead to reduction, increase or the complete suppression of gene expression and enzyme activity.

Most associations of genetic polymorphisms with ADRs relate to type A reactions but a few variants of CYP isozymes, UGTs, NATs and ABC transporters have been associated with idiosyncratic ADRs and may contribute to the individual susceptibility observed (table 1.2).

Table 1.2: Genetic polymorphisms associated with idiosyncratic hypersensitivity reactions

Drug	Drug class	ADR	Genetic variant	Reference
Phase I polymorphisms				
Clopidogrel	Antiplatelet drug	Cardiovascular ADRs	*CYP2C19*	(MEGA *et al.*, 2010)
Codeine	Opiate drug	Toxic systemic ADRs	*CYP2D6*	(GASCHE *et al.*, 2004)
Phase II polymorphisms				
Cisplatin	Chemotherapy drug	Hearing loss	*COMT* *TMPT*	(ROSS *et al.*, 2009)
Irinotecan	Chemotherapy drug	Neutropenia	*UGT1A1*	(IYER *et al.*, 2002)
Sulfamethoxazole	Antibiotic	Hypersensitivity	*GCLC* *NAT1*	(WANG *et al.*, 2012a) (WANG *et al.*, 2011a)
Thiopurine	Immunosuppressant	Hematologic toxicity	*TPMT*	(EVANS *et al.*, 1991)
Tolcapone	COMT inhibitor	Transient ALT elevations	*UGT1A*	(ACUNA *et al.*, 2002)
Troglitazone	Thiazolidinediones	Hepatic failure (ALT/AST elevations)	*GSTT1* *GSTM1*	(WATANABE *et al.*, 2003)
Phase III polymorphisms				
Cyclosporine	Immunosuppressant	Nephrotoxicity	*ABCB1*	(HAUSER *et al.*, 2005)
Statins	HMG-CoA reductase inhibitor	Myopathy	*SLCO1B1*	(LINK *et al.*, 2008)

List of associations shows only several relevant examples (adapted from: WEI *et al.*, 2012). *ABC* ATP-binding cassette, *COMT* catechol-O-methyl transferase, *CYP* cytochrome P450 enzyme, *DILI* drug-induced liver injury, *GCLC* glutamate cysteine ligase catalytic subunit, *NSAID* non-steroidal anti-inflammatory drug, *SLC* solute carrier, *TPMT* thiopurine methyl transferase

1.3.3 HLA-associations and drug-induced hypersensitivity

As described above, the interaction of MHC molecules and TCRs is crucial for the initiation of the adaptive immune response and the aetiology of drug-induced adverse reactions. This chapter examines the immunological background of these reactions and discusses the associations between HLA-alleles and the susceptibility to hypersensitivity.

In infectious diseases, certain aspects of disease protection and susceptibility have been associated to a number of class I and class II alleles. These include the association of various HLA-alleles and the progression to acquired immunodeficiency syndrome (AIDS) in HIV-1 infected individuals; rapid progression is significantly associated with HLA class I homozygosity and the *HLA-B*35, -C*04* alleles, whereas *HLA-B*57* has been found to be negatively associated with disease progression [table 1.4 (ALTFELD *et al.*, 2003; CARRINGTON *et al.*, 1999)]. Another example is reduced susceptibility to severe malaria in sub-Saharan populations [*HLA-B*53* and *HLA-DRB1*13:02-DQB1*05:02* haplotype (HILL *et al.*, 1991)].

Several studies have also identified strong links between specific HLA-alleles and hypersensitivity to various drugs, including antibiotics, antiretrovirals and non-steroidal anti-inflammatory drugs (summarised in table 1.3). Overall each drug appears to be associated with a specific set of HLA-alleles and the association is also dependent on the disease phenotype. Additionally the HLA-alleles linked to drug-induced ADRs are population-dependent, which may be based on the allele frequencies that can vary between different ethnicities.

This section will discuss some of the best-defined examples of drug-induced HSRs with associations to HLA-alleles (ABC, CBZ and allopurinol). As the focus of this thesis lies on nevirapine and its ability to induce HSRs, it will be reviewed in section 1.5 in more detail; a short representation of all known HLA associations and NVP-induced hypersensitivity can be found in table 1.6 below.

Table 1.3: Associations of HLA class I and II alleles with drug-induced hypersensitivity

Drug	Drug class	HLA-allele	Adverse reaction	Population	Reference
MHC class I associations					
Abacavir	Antiretroviral	*B*57:01*	DRESS/DIHS/HSS	Caucasians	(HETHERINGTON *et al.*, 2001; MALLAL *et al.*, 2002)
Allopurinol	Anti gout	*B*58:01*	SJS/TEN and DRESS/DIHS/HSS	Han Chinese Koreans Japanese Thai Caucasians	(HUNG *et al.*, 2005) (KANG *et al.*, 2011) (KANIWA *et al.*, 2008) (TASSANEEYAKUL *et al.*, 2009) (LONJOU *et al.*, 2008)
Carbamazepine	Antiepileptic	*B*15:02*	SJS/TEN	Han Chinese Thai Malay Indian	(CHUNG *et al.*, 2004) (TASSANEEYAKUL *et al.*, 2010) (CHANG *et al.*, 2011) (MEHTA *et al.*, 2009)
		*A*31:01*	DRESS/DIHS/HSS	Japanese Caucasians	(OZEKI *et al.*, 2011) (MCCORMACK *et al.*, 2011)
Feprazone	Analgesic	B22	Fixed-drug eruption	Italian	(PELLICANO *et al.*, 1997)
Flucloxacillin	Antibiotic	*B*57:01*	DILI	Caucasians	(DALY *et al.*, 2009)
Levamisole	Anthelminthic	*B*27*	Agranulocytosis	South American	(DIEZ, 1990)
Oxicam	NSAID	*A*2, B*12*	SJS/TEN	Caucasians	(ROUJEAU *et al.*, 1987)

Table 1.3: continued

Drug	Drug class	HLA-allele	Adverse reaction	Population	Reference
MHC class I associations					
Phenytoin	Antiepileptic	*B*15:02*	SJS/TEN	Han Chinese	(MAN *et al.*, 2007)
Sulfamethoxazole	Antibiotic	*A*30-B*13-Cw*6* haplotype	Fixed-drug eruption	Turkish	(OZKAYA-BAYAZIT and AKAR, 2001)
		*B*38*	SJS/TEN	Caucasians	(LONJOU *et al.*, 2008)
Sulfonamides	Antibiotic	*A*29, B*12, DR7*	SJS/TEN	Caucasians	(ROUJEAU *et al.*, 1987)
MHC class II associations					
Amoxicillin-clavulanate	Antibiotic	*DRB1*15:01*	DILI	Caucasians	(LUCENA *et al.*, 2011)
Aspirin	NSAID	*DPB1*03:01*	Asthma	Korean	(KIM *et al.*, 2008)
		*DRB1*13:02-DQB1*06:09* haplotype	Urticaria	Korean	(KIM *et al.*, 2005)
Efavirenz	Antiretroviral	*DRB1*01*	Delayed exanthema	French	(VITEZICA *et al.*, 2008)
Lapatinib	Anti-cancer	*DRB1*07:01-DQA1*02:01-DQB1*02:02/02:03*	DILI	Caucasians	(SPRAGGS *et al.*, 2011)
Lumiracoxib	Antiarthritic	*DRB1*15:01-DQA1*01:02* haplotype	DILI	Caucasians	(SINGER *et al.*, 2010)
NSAIDS		*DR*11*	Anaphylactic and cutaneous reactions	Spanish	(QUIRALTE *et al.*, 1999)
Ximelagatran	Antithrombotic agent	*DRB1*07, DQA1*02*	Elevated alanine aminotransferase	Caucasians	(KINDMARK *et al.*, 2008)

DRESS drug-induced eosinophilia and systemic reactions, *DIHS* drug-induced hypersensitivity syndrome, *HSS* hypersensitivity syndrome, *SJS* Stevens-Johnson syndrome, *TEN* toxic epidermal necrolysis, *DILI* drug-induced liver injury, *NSAID* non-steroidal anti-inflammatory drug

*Abacavir hypersensitivity syndrome and HLA-B*57:01*

Abacavir is a NRTI used in combination therapies for HIV-1 infections since 1999 (see section 1.3). Hypersensitivity to ABC occurs in approximately 5-8% of patients initiating treatment and is characterised by at least two of the following symptoms: rash, fever, nausea and gastrointestinal or respiratory symptoms. Reactions develop within six weeks after exposure and resolve upon withdrawal of the drug; rechallenge of patients however leads to the occurrence of more severe, life threatening reactions (CUTRELL *et al.*, 2004; HETHERINGTON *et al.*, 2001). The presence of CD8+ T cells in the skin of patients with ABC-induced rash implies that the pathogenic mechanism of ABC-induced HSRs are immune mediated (PHILLIPS *et al.*, 2002).

In 2002, two independent studies identified a strong association between ABC hypersensitivity and the MCH class I allele *HLA-B*57:01* (HETHERINGTON *et al.*, 2002; MALLAL *et al.*, 2002). Clinical over-diagnosis of ABC HSRs and ethnic variations in the *B*57:01* carriage frequency contributed to a low sensitivity of *HLA-B*57:01* screening for ABC-induced ADRs. Later, diagnosis of true immunologically mediated ABC hypersensitivity was improved through the introduction of patch testing and allowed clinical studies to evaluate the sensitivity of HLA-genotyping more clearly (HUGHES *et al.*, 2004; PHILLIPS *et al.*, 2002). The randomized double-blind controlled trial PREDICT-1 and the SHAPE case-control study demonstrated a negative predictive value of 100% and a positive predictive value of 47.9% of *HLA-B*57:01* for ABC hypersensitivity across various populations (MALLAL *et al.*, 2008). In response to these findings, which have subsequently been validated by a number of independent studies (RAUCH *et al.*, 2006; YOUNG *et al.*, 2008; ZUCMAN *et al.*, 2007), *HLA-B*57:01* screening is now mandated by the US Food and Drug Administration (FDA) prior to ABC prescription (FDA, 2008). Thus, the introduction of *HLA-B*57:01* genotyping into clinical practice reduced not only the incidence of ABC hypersensitivity from approximately 5-8% to < 1%, but also the number of cases of unnecessary withdrawal of ABC treatment [from 8% prior to screening to < 3% (BANNISTER *et al.*, 2008; RAUCH *et al.*, 2006; WATERS *et al.*, 2007; ZUCMAN *et al.*, 2007)].

Fifty per cent of *HLA-B*57:01* positive patients tolerate ABC treatment, thereby indicating that other factors such as genetic or environmental aspects, must affect the occurrence of ABC-induced hypersensitivity. The potential for inducing a CD8+ T cell mediated immune response in HIV-negative, *HLA-B*57:01* positive healthy volunteers argues against a contribution of HIV infection to the development of ABC-induced HSRs (CHESSMAN *et al.*, 2008). Further studies are needed to elucidate the contribution of different TCRs and regulatory T cell types toward the individual susceptibility of ABC-treated patients.

Several groups have shown that activation of ABC-specific T cells is dependent on the formation of direct bonds between ABC an endogenous MHC molecules (ILLING *et al.*, 2012; NORCROSS *et al.*, 2012; OSTROV *et al.*, 2012). As such, ABC binding changes the conformation of the MHC binding groove and alters the peptides loaded onto HLA-B*57:01. A differentiated T cell response is then triggered by the presentation of novel self-peptides through the ABC-modified MHC peptide complex.

*Carbamazepine hypersensitivity and HLA-B*15:02 or HLA-A*31:01*

Carbamazepine is an aromatic amine anticonvulsant primarily used in the treatment of epilepsy, bipolar disorders and trigeminal neuralgia that causes several different types of cADRs, including the life threatening reactions SJS and TEN. These reactions are characterised by severe epithelial cell apoptosis on epidermal and mucosal membranes, occurring within two months of treatment initiation. Despite being relatively rare in Europeans (one to six in 10.000 patients), the prevalence of CBZ-induced SJS/TEN is significantly higher in Chinese and Southeast Asians (CHUNG *et al.*, 2004; PHILLIPS and MALLAL, 2010).

The first study to report a strong association between *HLA-B*15:02* and CBZ-induced SJS/TEN in Han Chinese was published in 2004 (CHUNG *et al.*, 2004). All patients with CBZ-induced SJS/TEN were carriers of *HLA-B*15:02*, but only 3% of CBZ-tolerant, and 8.6% of the population controls were positive for the allele. This association has since been replicated not only in Han Chinese (MAN *et al.*, 2007; WANG *et al.*, 2011b; WU *et al.*, 2010; ZHANG *et al.*, 2011b), but also in Thai,

Malay and Indian populations (CHANG *et al.*, 2011; MEHTA *et al.*, 2009; TASSANEEYAKUL *et al.*, 2010). In CBZ-patients of Caucasian and Japanese ancestry, the association of *HLA-B*15:02* and CBZ-induced SJS/TEN has not been observed (IKEDA *et al.*, 2010; KANIWA *et al.*, 2008; LONJOU *et al.*, 2008). The *HLA-B*15:02* allele has a very low frequency in Caucasians and Japanese (< 0.01%) compared to people in southeast Asia, India and China explaining the high prevalence of SJS/TEN reactions in these specific populations (GONZALEZ-GALARZA *et al.*, 2011). A meta-analysis of *HLA-B*15:02* and CBZ-induced SJS/TEN in Asian patients showed that *HLA-B*15:02* positive patients are over a hundred times more likely to develop SJS/TEN during CBZ-treatment [OR: 113.39; confidence interval (95% CI) 51.24 – 250.97 (YIP *et al.*, 2012)]. In 2007, the U.S. FDA recommended *HLA-B*15:02* screening for all Asian patients "with ancestry from areas where *HLA-B*15:02* is present", prior to CBZ-treatment (FDA, 2007).

Various *in vitro* studies have shown that CD4+ and CD8+ T cells from patients can be stimulated by CBZ treatment and that usage of specific TCRs is important for CBZ-induced SJS/TEN in *HLA-B*15:02* positive individuals (KO *et al.*, 2011; WU *et al.*, 2007). Additional data suggest that the activation of T cells by HLA-B*15:02 is mediated via the p-i model: the interaction of the drug with the MHC/peptide complex occurs extracellularly without the involvement of intracellular CBZ-metabolism and can be abolished by the incubation with anti-HLA-B antibodies (BHARADWAJ *et al.*, 2012). Further, endogenous peptides loaded onto the MHC molecule were required to stabilise the MHC/peptide complex and facilitate CBZ binding. *In vivo* studies have shown that CD8+ T cells, able to induce keratinocyte-specific apoptosis, can be found in blister fluids of TEN patients supporting the role of T cell activation and immune response as part of the pathophysiological pathways leading to *HLA-B*15:02* associated CBZ-induced SJS/TEN (NASSIF *et al.*, 2004; NASSIF *et al.*, 2002).

Drug-induced SJS has been associated with several other aromatic amine anticonvulsants, e.g. oxcarbazepine and phenytoin (HUNG *et al.*, 2010; MAN *et al.*, 2007), as well as a number of different alleles that are members of the HLA-B75 serotype (IKEDA *et al.*, 2010; KANIWA *et al.*, 2010; KIM *et al.*, 2011). These results indicate that the pathophysiological mechanism of SJS/TEN caused by aromatic

amine anticonvulsants may be related to a possible cross-reactivity of these drugs and the activation of similar immunological pathways.

*HLA-A*31:01* was first reported as a possible risk allele for CBZ-induced MPE in a Han Chinese population by Hung *et al.* (2006). Later, carriage of *HLA-A*31:01* was linked to several phenotypes of CBZ hypersensitivity, including HSS and SJS/TEN in Caucasians, Japanese and Koreans (KASHIWAGI *et al.*, 2008; KIM *et al.*, 2011; MCCORMACK *et al.*, 2011; OZEKI *et al.*, 2011). The pooled meta-analysis of reported *HLA-A*31:01* associations with CBZ hypersensitivity throughout all populations generated an OR of 9.5 [95% CI 6.42 – 13.93 (YIP *et al.*, 2012)]. The allele frequency of *HLA-A*31:01* is more generally distributed with 2-7% in Han Chinese, 5% in Koreans, 7-12% in Japanese and 2-6% in western Europeans (GONZALEZ-GALARZA *et al.*, 2011). Whereas the pathogenic mechanism of CBZ-induced SJS/TEN and *HLA-B*15:02* associations has been carefully investigated, nothing is known about the mechanism underlying *HLA-A*31:01* associated HSRs.

The contribution of other genetic and environmental factors to the development of CBZ-induced HSRs cannot be excluded as *HLA-B*15:02* and *HLA-A*31:01* are not required nor sufficient for the development of HSRs and many patients carrying these alleles tolerate treatment with CBZ and other aromatic amine anticonvulsants.

*Allopurinol-induced hypersensitivity and HLA-B*58:01*

Allopurinol is a xanthine oxidase inhibitor known to cause cutaneous ADRs in 2% of patients (ARELLANO and SACRISTAN, 1993). First published in 2005 in a Han Chinese population from Taiwan (HUNG *et al.*, 2005), the strong association between allopurinol hypersensitivity and the *HLA-B*58:01* allele has since been replicated in Thai (TASSANEEYAKUL *et al.*, 2009) and Koreans (KANG *et al.*, 2011). In Japanese (DAINICHI *et al.*, 2007; KANIWA *et al.*, 2008) and Caucasians (LONJOU *et al.*, 2008) the association between *HLA-B*58:01* and allopurinol-induced ADRs is weaker than in the Southeast Asian populations, suggesting that the strength of the association may be connected to the HLA-allele frequencies in the different populations. The frequency of *HLA-B*58:01* is 2-11% in Han Chinese,

6-9% in Thai and 7% in Koreans whereas it is low in Caucasians (1-6%) and Japanese [0.5% (GONZALEZ-GALARZA *et al.*, 2011)].

As stated previously, *HLA-B*58:01* positive tolerant patients indicate that the presence of *HLA-B*58:01* is not sufficient for the occurrence of drug-induced HSRs in allopurinol-treated patients. No data on the pathogenic mechanism involved in the development of allopurinol-induced ADRs has been published to date.

1.4 Acquired immune deficiency syndrome

AIDS is the most extreme form of immune suppression observed in humans and is caused by HIV infection. Progression to AIDS is triggered by the gradual loss of the immune system whereby HIV infected individuals become susceptible to opportunistic infections through bacteria, fungi, parasites and viruses. AIDS-associated malignancies are now recognised as the most common cause of death, as HIV infected patients have a higher risk of developing aggressive forms of Kaposi's sarcoma as well as non-Hodgkin lymphoma and cervical cancer (reviewed in KALLINGS, 2008). Although first described in 1981, HIV infection was not recognised as the cause of AIDS until 1983 (BARRE-SINOUSSI *et al.*, 1983; CHERMANN *et al.*, 1983; GOTTLIEB *et al.*, 1981).

To date AIDS is epidemic, with prevalence rates as high as 18% across southern and eastern Africa. In 2012, an estimated 34 million people worldwide were HIV positive, of which 69% were living in sub-Saharan Africa. Nearly 3 million people were newly infected with HIV in 2010, although the annual incidence of HIV infections has fallen in 33 countries since 2001 (UNAIDS, 2012b; WHO, 2011).

1.4.1 Human immunodeficiency virus 1

Two types of HIV have been identified, namely HIV-1 and HIV-2 (CLAVEL *et al.*, 1986; REEVES and DOMS, 2002). Whereas HIV-2 infections are predominantly

restricted to West Africa, most occurrences of AIDS worldwide are caused by the more virulent type HIV-1 (KILMARX, 2009). Infection with HIV occurs through transmission of body fluids and is dependent on the concentration of both free virus and virus infected cells as well as the susceptibility of the host (LEVY, 1988). The most common route of infection is sexual intercourse but mother-to-child transmission (MTCT) is also observed.

Structure and molecular features

Mature HIV virions consist of a lipid bilayer membrane of host cell origin and a tetrameric envelope protein complex, encoded by the virus (see figure 1.6). The viral envelope protein complex is composed of two subunits, gp120 (or outer envelope protein) and gp41 (transmembrane protein). Like all retroviruses the lipid bilayer membrane surrounds the virus capsid, which contains the matrix protein p17, the major capsid protein p24, the nucleocapsid protein p7/p9, genomic RNA molecules and the three main viral enzymes (protease, reverse transcriptase (RT) and integrase).

The HIV genome consists of two identical 9.3 kb single stranded RNA molecules coding for nine genes. The coding region is flanked by two long terminal repeats (LTRs), which can bind host transcription factors and are required for the integration of the provirus into the host DNA. The *gag* gene encodes the structural HIV proteins (p17, p24 and p7/p9), while protease, integrase and RT are encoded by *pol*. The envelope precursor protein gp160, containing the two envelope proteins gp41 and gp120, is encoded by the *env* gene. In addition, HIV-1 contains the accessory genes *vif*, *vpr*, *rev* (regulator of expression); *tat* (transactivator of transcription); *vpu* and *nef* (negative regulatory factor) (BARRE-SINOUSSI, 1996; SIERRA *et al.*, 2005).

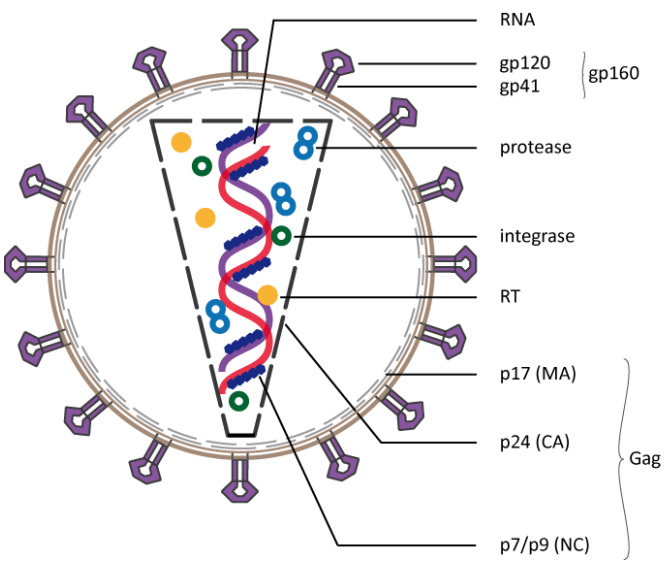


Fig. 1.6: Schematic representation of an HIV virion
Viral enzymes (protease, integrase and RT) as well as genomic RNA molecules associated with NC proteins are enclosed in the major capsid protein. The lipid bilayer membrane includes a tetrameric envelope protein complex consistent of the gp120 and pg41 subunits (Adapted from: BARRE-SINOUSSI, 1996; MURPHY *et al.*, 2012). *CA* capsid protein, *MA* matrix protein, *NC* nucleocapsid protein, *RT* reverse transcriptase

Replication cycle of HIV-1

HIV infection begins with the binding of the virus to the cell surface (figure 1.7). This is facilitated by the interaction of the HIV-1 gp120 with the cellular receptor CD4, which leads to the conformational change in gp120 uncovering a co-receptor binding site (SATTENTAU and MOORE, 1993; YOON *et al.*, 2010). The chemokine receptors CCR5 and CXCR4 are the main HIV co-receptors promoting fusion and entry to the host cell. R5 virus strains use the CCR5 for entry to macrophages, whereas X4 viruses use the CXCR4 receptor for infection of T cells [M- and T-tropic viruses (LEVY, 1988; MURAKAMI and YAMAMOTO, 2010)]. Binding to the co-receptors leads to the fusion of the viral and cellular membranes releasing the viral core into the cytoplasm.

Once uncoating of the virion core is completed the reverse transcription complex (consisting of the viral RNA, RT, integrase and several other viral proteins as well as a multitude of host proteins) is formed (FREED, 2001; SIERRA *et al.*, 2005). Within this complex, the viral RNA is retro-transcribed into double-stranded DNA and upon its completion the HIV provirus translocates to the nucleus. Integration of the provirus into the host genome is mediated by the viral integrase. The initiation of viral transcription leads to the production of multi-spliced transcripts of Nef, Tat and Rev through the activation of the LTR

promoter by the nuclear transcription factor κB (NF-κB). Tat controls efficient RNA-polymerase II (Pol II) elongation of viral transcription, while Ref promotes the transport of viral mRNA molecules into the cytosol. The viral protein Nef alters endosome trafficking thus reducing the cell surface expression of CD4 and MHC molecules (particularly HLA-A and HLA-B expression) and providing protection against immune-mediated cell death. Following the expression of Tat, Rev and Nef, single-spliced and unspliced mRNA molecules are expressed and transported out of the nucleus. The incorporation of both, the gp41 and gp120 precursor protein gp160 as well as Gag polyprotein into the cellular membrane leads to the initiation of HIV assembly. A ribosomal frameshift leads to the translation of the Gag-Pol polyprotein that includes the viral enzymes, such as protease, integrase and RT. The assembly of the immature virion core is completed once the genomic RNA, enzymes and structural proteins associate at the host membrane. Fusion of the cellular membrane around the core completes the budding process. During virus maturation, the protease auto-catalytically cleaves the Gag-Pol polyproteins, releasing the viral enzymes into the core (FREED, 2001; SIERRA *et al.*, 2005; TRKOLA, 2004).

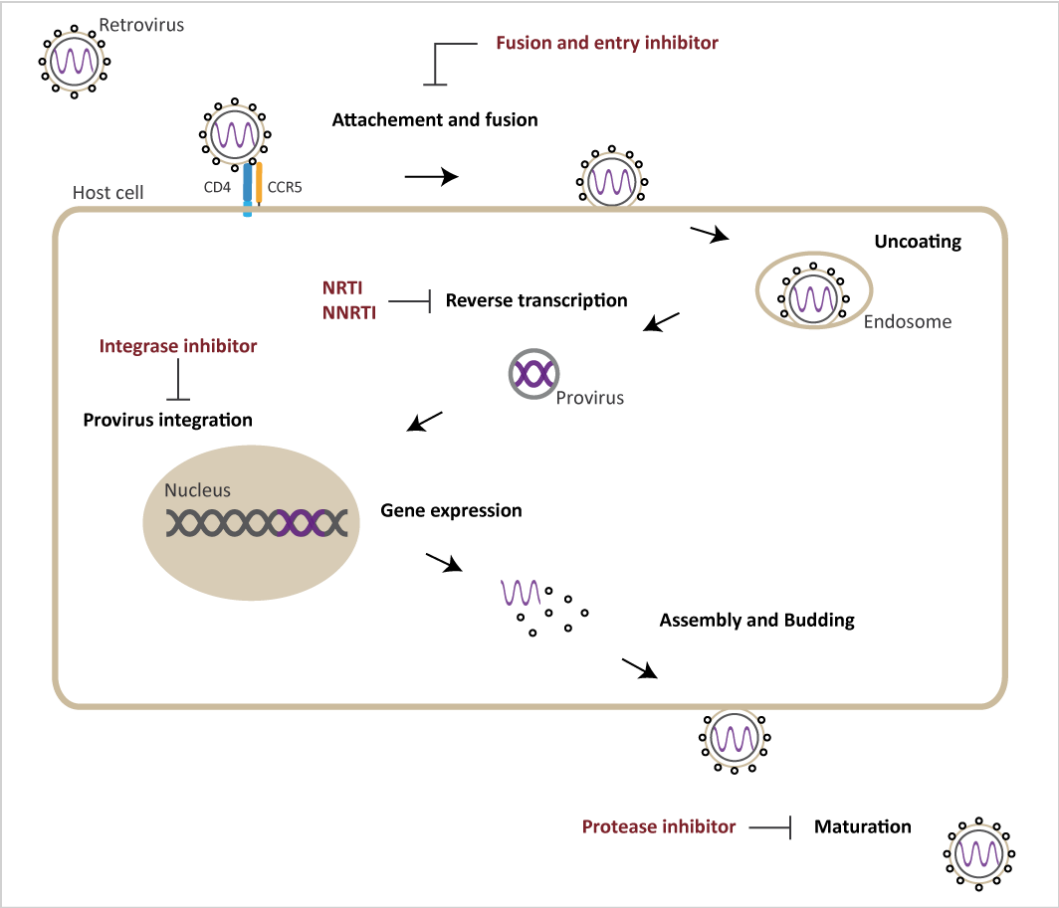


Fig. 1.7: HIV-1 replication cycle and targets of antiretrovirals
Binding of HIV virion to CD4 receptor and CCR5 or CXCR4 co-receptor promotes membrane fusion and entry of the virion core into the host cell. Following uncoating, reverse transcription of the viral genomic RNA is initiated and the provirus translocates towards the nucleus. Integration of the provirus into the host genome induces the production of viral messenger RNAs. The viral proteins Tat, Nef and Rev are translated from multi-spliced mRNA molecules. Single-spliced mRNA molecules are translated into envelope proteins, whereas full-length mRNAs provide new viral genomic RNA as well as templates for the core proteins. Viral core proteins and enzyme precursors assemble at the host cell membrane forming the immature virion core. Subsequently immature virions are released from the cells and the processing of precursor proteins by the viral protease initiates virus maturation. The molecular targets of antiretroviral therapy are indicated in red (adapted from: BARRE-SINOUSSI, 1996). *NRTI* nucleoside and nucleotide reverse transcriptase inhibitor, *NNRTI* non-nucleoside reverse transcriptase inhibitor

Immune response in HIV-1 infection

HIV infection can be divided into three stages: the acute or primary infection is characterised by influenza-like symptoms, increase in viremia and loss of CD4+ T cells. It occurs two to four weeks after infection and the initiation of an HIV-specific cellular immune response leads to HIV antibody production, known as seroconversion and a decline in the plasma viral load. During the clinical

asymptomatic stage, which can last for about ten years, people remain infectious and persistent HIV replication in the lymph nodes leads progressively to a loss of CD4+ T cells. Once the decline in CD4+ T cells reaches a critical point (< 200 cells/µl) and cell-mediated immunity is lost, the occurrence of opportunistic infections increases, which indicates the progression from HIV infection to AIDS.

The cellular immune response during primary infection is characterised by the activation of cytotoxic CD8+ T cells (CTLs) prior to seroconversion. Recognition of HIV infected cells requires the interaction of MHC molecules associated with viral peptides and the TCR, which leads to the lysis of infected cells. Besides the CTL mediated killing of HIV infected cells, binding of CTLs induces the release of chemokines that are endogenous ligands to the CCR5 co-receptor and inhibit CCR5 mediated fusion of the virus and the CD4+ T cell membrane. Neutralizing antibodies targeted against the extracellular domains gp120 and gp41 mediate the phagocytosis of virions as part of humoral immunity. However both, the cellular as well as the humoral immune responses are unable to eradicate the virus and clear the infection.

This is favoured by high mutation rates of HIV-1, which allows the virus to escape CTL recognition through mutations within viral antigens and overcome the antiviral activity of neutralising antibodies trough conformational changes of mutated gp120 molecules (reviewed in: MURPHY *et al.*, 2012; SIERRA *et al.*, 2005).

Immune control of HIV-1 infection by the host

Untreated HIV-1 infected individuals have shown differences in disease outcome, with the majority of patients progressing to AIDS in less than a year. Nevertheless, approximately 5% of infected individuals (also called long term nonprogressors) sustain normal CD4+ T cell counts and low virus titres for decades (CAO *et al.*, 1995). Patients who sustain undetectable HIV plasma viral load for longer than ten years represent less than 1% of HIV infected individuals and are known as elite controllers or HIV controllers (SAEZ-CIRION *et al.*, 2007).

Long-term survival without antiretroviral therapy (ART) is determined by the following factors (LEVY, 2009; SAEZ-CIRION *et al.*, 2007):

(a) mutations in the infecting virus strain itself (e.g. lack of Nef);
(b) strong T cell mediated immune response and high amount of neutralising antibodies;
(c) the immunogenic background of the host (discussed below).

A deletion of 32 base pairs (bp) in the gene encoding the CCR5 co-receptor (CCR5Δ32) leads to the formation of a truncated protein (DEAN *et al.*, 1996). This protein, which is not expressed on the cell surface, has been shown to actively downregulate CXCR4 expression and individuals homozygous for the mutant CCR5Δ32 allele are highly resistant against HIV-1 infection (AGRAWAL *et al.*, 2007; AGRAWAL *et al.*, 2004). In heterozygous patients, progression to AIDS is delayed (MEYER *et al.*, 1997; O'BRIEN and MOORE, 2000). The CCR5Δ32 mediated protection is not absolute: homozygous CCR5Δ32 individuals can be infected by the X4 virus strain, leading to a persistent HIV-1 infection (O'BRIEN *et al.*, 1997; SHEPPARD *et al.*, 2002).

As described above, a number of HLA-alleles have been associated with protective effects or higher susceptibility in infectious diseases (see table 1.4). Recently these associations were replicated in a multinational consortium, which established that immunogenic control of HIV-1 by the host was due to specific amino acids of the HLA-B binding groove. Changes of these key residues may affect HLA-allele specific binding properties and MHC mediated peptide presentation in HIV infected cells. Also variation in HLA-C expression (associated with the SNP rs9264942) may affect the host response to HIV (INTERNATIONAL HIV CONTROLLERS STUDY *et al.*, 2010).

Table 1.4: Most consistent associations of HLA-alleles and HIV-1 progression

HLA locus	Reference
Rapid progression to AIDS	
MHC class I homozygosity (One gene locus)	(CARRINGTON *et al.*, 1999; TANG *et al.*, 1999)
*HLA-B*35*	(CARRINGTON *et al.*, 1999; ITESCU *et al.*, 1992)
Delay in AIDS progression	
*HLA-B*27*	(HENDEL *et al.*, 1999; KASLOW *et al.*, 1996)
*HLA-B*57*	(COSTELLO *et al.*, 1999; HENDEL *et al.*, 1999; KASLOW *et al.*, 1996)

MicroRNA mediated regulation of HLA-C expression

First described in 2009, a SNP located 35 kb upstream of the *HLA-C* locus was associated with the viral plasma load and *HLA-C* mRNA levels (INTERNATIONAL HIV CONTROLLERS STUDY *et al.*, 2010; THOMAS *et al.*, 2009). The -35 SNP was later shown to be in strong LD with the microRNA miR-148a binding site in the *HLA-C* 3' untranslated region [UTR (KULKARNI *et al.*, 2011)].

MicroRNAs (miRNAs) are highly conserved ~22 nucleotide-long RNA molecules known to post-transcriptionally regulate gene expression. The miR-148a mediated regulation of HLA-C surface expression is controlled by a single bp insertion/deletion of position 263 located downstream of the *HLA-C* stop codon (263del). This deletion lies within the miRNA-binding site and prevents miR-148a binding to the 3' UTR of *HLA-C*. Alleles that contain 263del (*HLA-C*02, C*05, C*06, C*08, C*12, C*15* and *C*16*) are therefore termed "escaped alleles" whereas miR-148a restricts the expression of alleles that have an intact miR-148a binding site ["inhibited alleles": *HLA-C*01, C*03, C*04, C*07, C*14* and *C*17* (KULKARNI *et al.*, 2011; O'HUIGIN *et al.*, 2011)]. Even though miR-148a has been shown to regulate *HLA-C* expression directly, other factors may contribute to the difference in allelic-specific expression patterns. Apps *et al.* have proposed that the HIV protection provided through an increase in HLA-C expression levels may be based on an intensified CTL mediated immune reaction (APPS *et al.*, 2013).

1.4.2 Antiretroviral therapy

The number of AIDS-related deaths has been declining in the past decade as ART became more widely accessible for people around the world. At present, eight million people have been reported to receive ART and also the number of HIV infected women receiving ART for the prevention of MTCT has been steadily increasing (UNAIDS, 2012b).

Currently, 25 antiretroviral drugs and various fixed-dose drug combinations have been approved for the treatment of HIV infections [table 1.5 (FDA, 2013)]. Antiretrovirals are categorized according to the specific step in the HIV replication cycle targeted by the drug (see figure 1.7) and aim to reduce the plasma viral load to undetectable levels and attain a normal immunological function through increase of the CD4+ T cell count. It has been suggested that patients with symptomatic HIV disease start therapy directly, whereas asymptomatic patients should not be initiated unless their CD4+ T cell count drops below 350 cells/µl and the risk for disease progression as well as possible comorbidities have been assessed (HAMMER *et al.*, 2008; VOLBERDING and DEEKS, 2010). Highly active antiretroviral therapy (HAART), a combination of two or three antiretrovirals, has achieved the best clinical benefits. The most common drug combinations consist of two NRTIs and either a non-nucleoside reverse transcriptase inhibitor (NNRTI), a protease inhibitor (PI) or an integrase inhibitor. The initial drug regimen may be changed if the patient experiences drug-induced ADRs or treatment fails to reduce the viral plasma load within 24 weeks of therapy (TSIBRIS and HIRSCH, 2010).

Nucleoside and nucleotide reverse transcriptase inhibitors

NRTIs were the first clinically used antiretroviral agents (YARCHOAN *et al.*, 1986). This class of drugs act as competitive inhibitors of the viral RT by binding to its catalytic site and preventing the incorporation of endogenous deoxynucleoside triphosphates (dNTPs) into the growing proviral DNA molecule. Similar to dNTPs, NRTIs require phosphorylation to form the active 5'-triphosphate molecule.

Non-nucleoside reverse transcriptase inhibitors

In contrast to NRTIs, binding of NNRTIs does not occur at the active site of the viral RT but in close proximity thus disrupting the interaction of the enzyme with its normal substrate. NNRTIs are HIV-1 specific but mutations of the amino acids with which these drugs interact are known to cause resistance of the virus to one or more drugs of this class. They are primarily metabolised via the hepatic pathway and are substrates for CYP enzymes.

Protease inhibitors

PIs selectively bind to the homodimeric structure of the HIV protease and inhibit the catalytic function of the enzyme, as they cannot be proteolytically cleaved. Ritonavir is often used at sub-therapeutic doses to increase the drug concentrations of other PIs as it also inhibits CYP3A4 function.

HIV integrase strand transfer inhibitor

HIV integrase inhibitors block the integration of the viral DNA into the host genome by preventing the strand transfer reaction of the HIV-1 and HIV-2 integrase.

Fusion and entry inhibitors

Fusion and entry inhibitors prevent the binding, fusion and entry of the virion to the host cell through the interaction with the viral gp41 glycoprotein or as a CCR5 co-receptor antagonist. CCR5 antagonists are only active against the R5 virus strain and require genotypic testing of virus tropism to avoid selective replication of X4 viruses (reviewed in: DE CLERCQ, 2009; TSIBRIS and HIRSCH, 2010).

Table 1.5: Antiretroviral drugs approved by the U.S. FDA

Generic Name	Abbreviation	Brand Name	Manufacturer	Approval Date
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)				
Abacavir	ABC	Ziagen	GlaxoSmithKline	17-Dec-1998
Didanosine	ddI	Videx EC Videx	Bristol Myers-Squibb	31-Oct-2000 9-Oct-1991
Emtricitabine	3TC	Emtriva	Gilead Sciences	2-Jul-2003
Lamivudine		Epivir	GlaxoSmithKline	17-Nov-1995
Stavudine	d4T	Zerit	Bristol Myers-Squibb	24-Jun-1994
Tenofovir disoproxil fumarate	TDF	Viread	Gilead	26-Oct-2001
Zidovudine	ZDV	Retrovir	GlaxoSmithKline	19-Mar-1987
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)				
Delavirdine	DLV	Rescriptor	Pfizer	4-Apr-1997
Efavirenz	EFV	Sustiva	Bristol Myers-Squibb	17-Sep-1998
Etravirine		Intelence	Tibotec Therapeutics	18-Jan-2008
Nevirapine	NVP	Viramune	Boehringer-Ingelheim	21-Jun-1996
Rilpivirine		Edurant	Tibotec Therapeutics	20-May-2011
Protease inhibitors (PIs)				
Atazanavir sulfate	ATV	Reyataz	Bristol-Myers Squibb	20-Jun-2003
Darunavir		Prezista	Tibotec, Inc.	23-Jun-2006
Fosamprenavir Calcium	FOS-APV	Lexiva	GlaxoSmithKline	20-Oct-2003
Indinavir	IDV	Crixivan	Merck	13-Mar-1996
lopinavir and ritonavir	LPV/RTV	Kaletra	Abbott Laboratories	15-Sep-2000
Nelfinavir mesylate	NFV	Viracept	Agouron Pharmaceuticals	14-Mar-1997
Ritonavir	RTV	Norvir	Abbott Laboratories	1-Mar-1996
Saquinavir mesylate	SQV	Invirase	Hoffmann-La Roche	6-Dec-1995
Tipranavir	TPV	Aptivus	Boehringer Ingelheim	22-Jun-2005

Table 1.5: continued

Generic Name	Abbreviation	Brand Name	Manufacturer	Approval Date
HIV integrase strand transfer inhibitors				
Dolutegravir		Tivicay	GlaxoSmithKline	13-Aug-2013
Ealtegravir		Isentress	Merck & Co., Inc.	12-Oct-2007
Fusion inhibitor				
Enfuvirtide	T-20	Fuzeon	Hoffmann-La Roche & Trimeris	13-Mar-2003
Entry inhibitor - CCR5 Receptor antagonist				
Maraviroc		Selzentry	Pfizer	6-Aug-2007
Fixed dose drug combinations				
Abacavir and lamivudine		Epzicom	GlaxoSmithKline	2-Aug-2004
Abacavir, zidovudine and lamivudine		Trizivir	GlaxoSmithKline	14-Nov-2000
Efavirenz, emtricitabine and tenofovir disoproxil fumarate		Atripla	Bristol-Myers Squibb and Gilead Sciences	12-Jul-2006
Elvitegravir, cobicistat, emtricitabine and tenofovir disoproxil fumarate		Stribild	Gilead Sciences	27-Aug-2012
Emtricitabine, rilpivirine and tenofovir disoproxil fumarate		Complera	Gilead Sciences	10-Aug-2011
Lamivudine and zidovudine		Combivir	GlaxoSmithKline	27-Sep-1997
tenofovir disoproxil fumarate and emtricitabine		Truvada	Gilead Sciences, Inc.	2-Aug-2004

1.5 Nevirapine

In 1996, NVP [Viramune©, Boehringer-Ingelheim (figure 1.8)] became the first NNRTI approved for the treatment of HIV-1 infections. It has been shown to reduce plasma viral load effectively as part of triple combination therapy and as a single-dose treatment for the prevention of MTCT. At steady state, the therapeutic range of NVP plasma concentration covers 11-30 µM after administration of 400 mg per day (BOEHRINGER-INGELHEIM, 2012). NNRTIs bind to a hydrophobic pocket near the dNTP and NRTI binding site. This leads to conformational modifications of enzyme structure, thereby inhibiting DNA-polymerase activity (TANTILLO *et al.*, 1994). Single bp mutations leading to amino acid changes in the binding domain, such as p.Y181C and p.K103N, reduce NNRTI-binding affinity and cause the expansion of NVP-resistant HIV-1 strains (DEEKS, 2001; RICHMAN *et al.*, 1994). Higher plasma levels of NVP have been associated with improved HIV clearance and a decrease in the occurrence of NVP-resistance mutations (GONZALEZ DE REQUENA *et al.*, 2005; VELDKAMP *et al.*, 2001).

1.5.1 Nevirapine pharmacokinetics

After oral administration, NVP is almost completely absorbed in the intestine with a mean bioavailability of more than 90%. Four hours after administration, maximum NVP plasma concentration is achieved. It is distributed throughout the body with only 60% of the administered drug binding to plasma proteins. In the liver, NVP is predominantly metabolised by CYP isoenzymes 2B6 and 3A4. Multiple dosing with 200–400 mg NVP per day results in auto-induction of these enzymes and leads to an increase in plasma clearance as well as a decrease in its half-life to 30 hours (discussed in: COOPER and VAN HEESWIJK, 2007). Nevirapine is primarily excreted in urine after glucoronide conjugation via the urine (RISKA *et al.*, 1999a).

Nevirapine metabolism

In both humans and rats, NVP is metabolised into 2-hydroxynevirapine (2-OH-NVP), 3-hydroxynevirapine (3-OH-NVP), 8-hydroxynevirapine (8-OH-NVP), 12-hydroxynevirapine (12-OH-NVP) and 4-carboxynevirapine (4-COOH-NVP) (RISKA *et al.*, 1999a; RISKA *et al.*, 1999b). While the formation of 2-OH- and 12-OH-NVP is primarily catalysed by CYP3A4, CYP2B6 plays a role in the biotransformation of 3-OH- and 8-OH-NVP (ERICKSON *et al.*, 1999). 12-OH-NVP is further oxidized to generate 4-COOH-NVP. Glucuronidation results in the excretion of these metabolites through the renal system.

Polymorphisms in genes encoding particular NVP-metabolising enzymes have been shown to influence NVP pharmacokinetics. The *CYP2B6* c.516G>T (rs3745274) polymorphism is associated with a decrease in the catalytic activity and affects NVP plasma concentrations in Black Africans (BROWN *et al.*, 2012; CICCACCI *et al.*, 2010; DICKINSON *et al.*, 2013; PENZAK *et al.*, 2007), Cambodian (CHOU *et al.*, 2010), Indian (RAMACHANDRAN *et al.*, 2009), Swiss (ROTGER *et al.*, 2005) and mixed populations (HEIL *et al.*, 2012; MAHUNGU *et al.*, 2009; WYEN *et al.*, 2008). Accordingly, the c.983T>C SNP (rs28399499) in *CYP2B6*, which is absent in Caucasian populations, has been associated with differences in NVP plasma levels (DICKINSON *et al.*, 2013; WYEN *et al.*, 2008). Recently, a SNP (rs2125739) in the ABCC11 efflux transporter was independently associated with NVP plasma concentrations (LIPTROTT *et al.*, 2012). Other factors that influence NVP plasma levels are ethnicity, gender, weight, concomitant hepatic disease and co-medication (HALL and MACGREGOR, 2007; LAMORDE *et al.*, 2011; STOHR *et al.*, 2008). Potential bioactivation pathways of NVP involve the substitution of the C-3 or C-12 through the formation of quinone methides or reactive epoxide intermediates leading to DNA or protein adducts, which have an immunogenic potential [figure 1.11 (SHARMA *et al.*, 2012; SHARMA *et al.*, 2013b)].

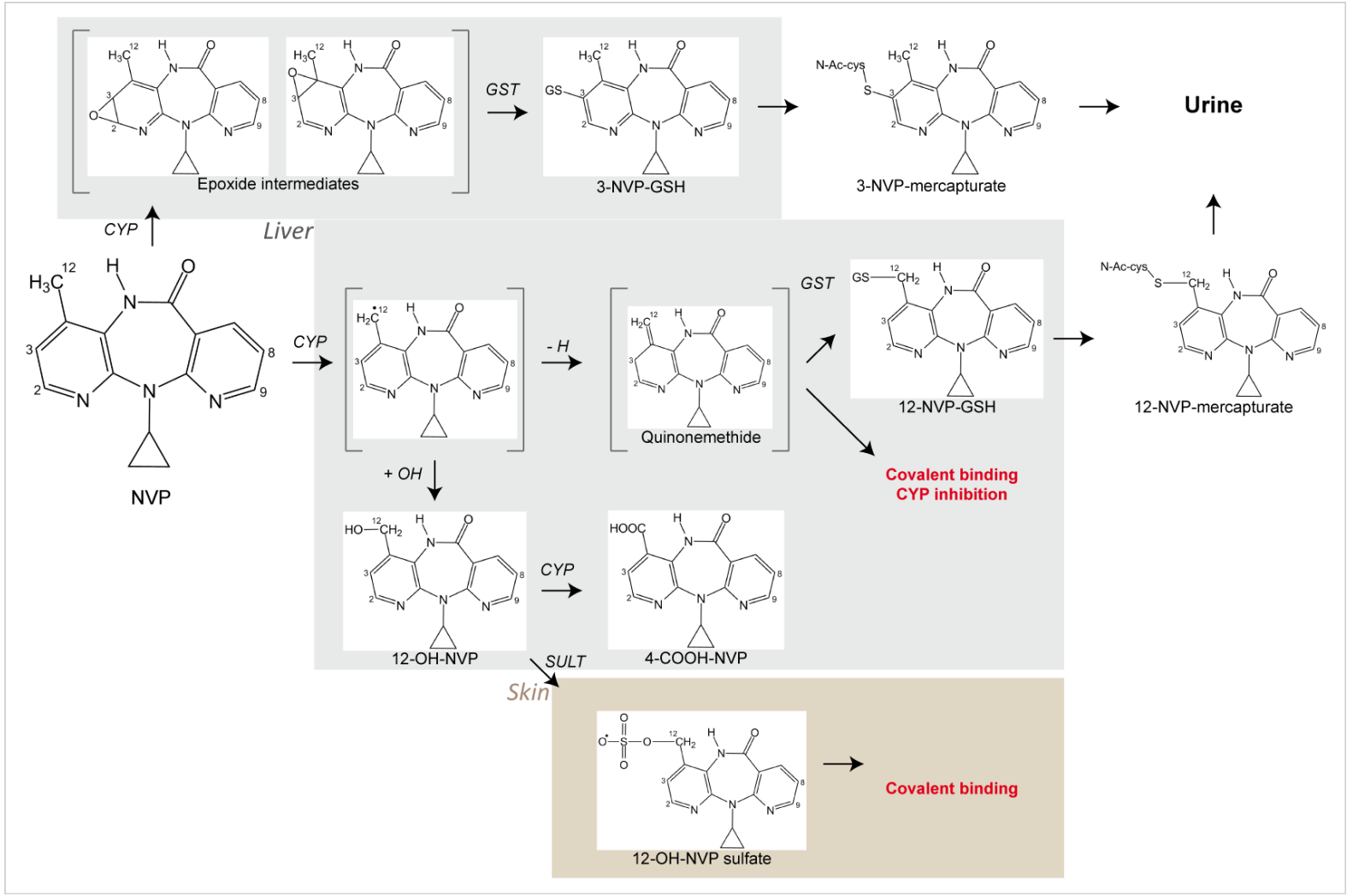


Fig. 1.8: Proposed pathway for the bioactivation of nevirapine

CYP cytochrome P459 enzyme, *GST* Gluthatione S-transferase, *NVP* nevirapine, *SULT* Sulfotransferase

1.5.2 Nevirapine-induced hypersensitivity reactions

Even though NVP-containing regimens are generally well tolerated, approximately 5% of NVP-treated patients develop HSRs within the first six weeks of medication. Nevirapine can cause different types of reactions, including combinations of rash, fever and hepatotoxicity. Rechallenge with NVP leads to rapid occurrence of more severe reactions (GANGAR *et al.*, 2000; POLLARD *et al.*, 1998).

An increased risk of rash-associated hepatotoxicity has been associated with higher pretreatment CD4+ T cell count in women (> 250 cells/µl) and men [> 400 cells/µl (BOEHRINGER-INGELHEIM, 2012; TAIWO, 2006)]. Accordingly, NVP is not recommended for postexposure prophylaxis of non-HIV infected individuals with higher CD4+ T cell counts (PATEL *et al.*, 2004; WIT *et al.*, 2008). Subsequent reports have confirmed this association (LYONS *et al.*, 2006; STERN *et al.*, 2003), while others have not found an association between NVP-induced hypersensitivity and CD4+ T cell counts (KNOBEL *et al.*, 2008; PETERS *et al.*, 2010). Additional risk factors associated with NVP-induced HSRs are listed in table 1.6. No direct correlation between NVP plasma levels and HSRs has been reported (WIT *et al.*, 2008).

As with other drug-induced HSRs, NVP hypersensitivity has been associated with various genetic polymorphisms (table 1.8) and HLA-alleles (table 1.7) that will be discussed in more detail below.

Table 1.6: Risk factors for nevirapine-induced hypersensitivity

Risk factor	Type of reaction
Abnormal ALT/AST levels at baseline	Hepatotoxicity
Female gender	Rash
HBV and/or HCV co-infection	Hepatotoxicity
Detectable plasma viral load in ART-experienced patients	Hypersensitivity
Concomitant use of alcohol, methadone and isoniazid	Hepatotoxicity

(Adapted from: BERSOFF-MATCHA *et al.*, 2001; TAIWO, 2006; TORTI *et al.*, 2007; WIT *et al.*, 2008)

Cutaneous adverse reactions

Rash is the most common adverse reaction observed in approximately 35% of NVP patients initiating therapy at 400 mg per day (BARNER and MYERS, 1998; HAVLIR *et al.*, 1996). The majority of these rashes are mild and lack the involvement of mucous membranes and systemic symptoms. More severe reactions can manifest as DRESS and in 0.3% of cases as SJS or TEN (POLLARD *et al.*, 1998; TAIWO, 2006). A lower lead-in dose of 200 mg NVP per day for the first two weeks of therapy, followed by 400 mg per day, has been shown to reduce the incidence of rash to 17% (CHEESEMAN *et al.*, 1993; POLLARD *et al.*, 1998). However, patients initiating NVP-therapy at lower concentrations are still at risk of developing NVP-induced cutaneous ADRs (FAGOT *et al.*, 2001; METRY *et al.*, 2001). Severe rash is more common in women than in men (BERSOFF-MATCHA *et al.*, 2001; TAIWO, 2006).

Only a few animal models have addressed the specificity of idiosyncratic drug reactions in humans. Nevirapine-induced skin rash in rats has very similar characteristics to the mild from of rash observed in patients. Female Brown Norway rats are more susceptible to rash than males and it takes 2-3 weeks for the rash to develop. Rechallenge results in a rapid onset of a more severe reaction. Depletion of CD4+ T cells confers partial protection against rash in rats, which is consistent with the observation that patients with lower CD4+ T cell counts are less likely to develop HSRs (SHENTON *et al.*, 2005; SHENTON *et al.*, 2003). This model further supports the hypothesis that the NVP-induced rash is immune-mediated. Hydroxylation of NVP, leading to the formation of 12-OH-NVP, is the metabolic pathway responsible for the development rash in rats (CHEN *et al.*, 2008a). It causes the formation of a benzylic sulphate, which binds covalently to cytosolic proteins thus initiating an active immune response and may indicate that the underlying mechanism of NVP-induced skin rash in rats is based on the hapten and danger hypotheses [figure 1.11 (SHARMA *et al.*, 2013a; SHARMA *et al.*, 2013b)]. The immune-mediated reaction observed in rats is characterised by the upregulation of either intracellular adhesion molecule (ICAM)-1 or MHC class II receptors and infiltration of macrophages into the

dermis. Additionally, lymphocyte infiltration and elevated cytokine levels were detected in NVP-treated animals (CHEN *et al.*, 2009).

Likewise in patients, lesions in the epidermis and dermis are strongly infiltrated by CD4+ and CD8+ T cells (NASSIF *et al.*, 2002; PICHLER, 2003). MPE patients show a strong migration of CD4+ T cells to the epidermis, whereas CD8+ T cells are more numerous in patients with SJS/TEN (HARI *et al.*, 2001; WETTER and CAMILLERI, 2010; YAWALKAR *et al.*, 2000). Macrophages were also found in the dermal infiltrate and may enhance the pro-inflammatory response through phagocytosis and antigen-presentation (PAQUET and PIERARD, 2002). Besides the cytotoxic properties of CD8+ T cells, the release of various cytokines and chemotactic molecules by CD4+ T cells and activated macrophages may mobilise neutrophils, thus contributing to epidermal cell death and inflammatory response (CAPRONI *et al.*, 2006; PICHLER *et al.*, 2002).

NVP-associated hepatotoxicity

Hepatotoxicity has been associated with all classes of antiretrovirals and can be classified as less severe events with abnormal elevations of ALT/AST levels (> 5x upper limit of normal (ULN)) in the presence or absence of symptoms of liver injury. Five to ten per cent of HIV-positive patients on HAART show asymptomatic elevations in ALT/AST levels with no difference in the incidence of NVP-induced elevations compared to other NNRTIs (STERN *et al.*, 2003). Asymptomatic ALT/AST elevations represent transient events that resolve spontaneously. Symptomatic elevations of ALT/AST are associated with systemic symptoms such as nausea, abdominal pain, fever and rash. These reactions occur in 4.9% of NVP-hypersensitive patients of whom 2.2% develop rash-associated symptomatic hepatic events (DIETERICH *et al.*, 2004) and require discontinuation of NVP treatment. Hepatic failure, the most severe case of drug-induced hepatotoxicity, occurs in 0.3% of NVP-treated patients and is characterised as progressive liver injury associated with elevated ALT levels, systemic symptoms as well as jaundice (DIETERICH *et al.*, 2004; TAIWO, 2006). Associated risk factors comprise alcohol use, HBV or HCV co-infections and high

baseline ALT/AST levels whereas the associations of elevated CD4+ T cell counts and gender were inconsistent (DIETERICH *et al.*, 2004; STERN *et al.*, 2003).

1.5.3 Pharmacogenetics of nevirapine-induced hypersensitivity

Based on the observation that NVP hypersensitivity is essentially dose-independent and lower CD4+ T cell count at treatment initiation can be protective in some patients, the majority of pharmacogenetic studies have focused on the MHC gene loci.

In contrast to the ABC hypersensitivity syndrome, a number of both MHC class I and class II alleles have been associated with NVP-induced HSRs in different populations (summarised in table 1.6). First reported in a Western Australian population, *HLA-DRB1*01:01* in combination with high CD4+ T cell counts was associated with the occurrence of hepatotoxicity (MARTIN *et al.*, 2005). Subsequently, several other alleles of the *DRB1*01* family have been associated with cutaneous and hepatic reactions in populations originating from Europe and Western Australia [*DRB1*01* (VITEZICA *et al.*, 2008; YUAN *et al.*, 2011), *DRB1*01:01* (PHILLIPS *et al.*, 2011) and *DRB1*01:02* (PHILLIPS *et al.*, 2013)]. MHC class I alleles belonging to the *B*35* family have been primarily implicated in NVP-induced cADRs in Caucasians [*B*35:01* (PHILLIPS *et al.*, 2011)], Indians [*B*35* (CHANTARANGSU *et al.*, 2009)] and Thai [*B*35* and *B*35:05* (UMAPATHY *et al.*, 2011; YUAN *et al.*, 2011)], whereas *B*58:01* has been associated with hepatotoxicity in a mixed population from Europe (PHILLIPS *et al.*, 2013). More recently, various groups have indicated a strong association between *HLA-C*04* and NVP-induced hypersensitivity and cADRs in Asians, Black Africans, Caucasians, Han Chinese and Thai (CARR *et al.*, 2013; GAO *et al.*, 2012; LIKANONSAKUL *et al.*, 2009; YUAN *et al.*, 2011). The association of *HLA-C*08* and the *C*08-B*14* haplotype with NVP hypersensitivity was described in a Japanese and a Sardinian population, respectively (GATANAGA *et al.*, 2007; LITTERA *et al.*, 2006). Later, the *B*35-C*04* and *B*15-DR*15* haplotypes were associated with HSRs in NVP-treated patients from Southeast Asia and Western Australia

(PHILLIPS *et al.*, 2011; YUAN *et al.*, 2011). Similar to CBZ, NVP hypersensitivity has been associated with different MHC alleles across various populations yet these associations appear to be phenotype specific. Whether the differences in allele frequencies may help to explain the diverse associations has not been investigated.

Table 1.7: HLA-alleles associated with an increased risk of nevirapine-induced hypersensitivity

HLA-allele	Adverse reaction	Population	OR	Reference
*B*15-DR*15* haplotype	Fever and hepatitis and/or rash	Caucasians	8.9	(PHILLIPS *et al.*, 2011)
*B*35*	Cutaneous adverse events/concomitant hepatic events	Thai	5.7	(YUAN *et al.*, 2011)
	Skin rash	Indian	3.4	(UMAPATHY *et al.*, 2011)
*B*35-C*04* haplotype	Cutaneous adverse events/concomitant hepatic events	Asians	18.4	(YUAN *et al.*, 2011)
		Thai	13.4	
*B*35:01*	Fever and hepatitis and/or rash	Caucasians	5.4	(PHILLIPS *et al.*, 2011)
*B*35:05*	Skin rash	Thai	19.0	(CHANTARANGSU *et al.*, 2009)
*B*58:01*	Hepatotoxicity	Mixed	3.2	(PHILLIPS *et al.*, 2013)
*C*04*	Cutaneous adverse events/concomitant hepatic events	Asians	2.6	(YUAN *et al.*, 2011)
		Blacks	5.2	
		Whites	1.9	
	Skin rash	Thai	2.8	(LIKANONSAKUL *et al.*, 2009)
	Hypersensitivity	Han Chinese	3.6	(GAO *et al.*, 2012)
*C*04:01*	SJS/TEN	Malawians	17.5	(CARR *et al.*, 2013)
	Hypersensitivity	Malawians	2.8	
*C*08*	Skin rash and/or hepatotoxicity	Japanese	6.2	(GATANAGA *et al.*, 2007)
*C*08-B*14* haplotype	Skin rash and/or hepatotoxicity with systemic reactions	Sardinian	NA	(LITTERA *et al.*, 2006)
*DRB1*01*	cADRs	Caucasians	70.0	(VITEZICA *et al.*, 2008)
	Hepatic adverse events	Whites	3.0	(YUAN *et al.*, 2011)
*DRB1*01:01* & CD4+ T cells > 25%	Fever and hepatitis and/or rash	Caucasians	8.9	(PHILLIPS *et al.*, 2011)
	Hepatotoxicity with multi-system reactions	Caucasians	5.5	(MARTIN *et al.*, 2005)
*DRB1*01:02*	Hepatotoxicity	Mixed	4.6	(PHILLIPS *et al.*, 2013)

Definitions of adverse reactions as stated by the authors. *cADR* cutaneous adverse drug reaction, *HLA* human leukocyte antigen, *OR* odds ratio, *SJS* Stevens-Johnson syndrome, *TEN* toxic epidermal necrolysis

A small number of groups have investigated the associations of NVP hypersensitivity and genetic polymorphisms in DMEs and drug transporters (table 1.8). Two studies reported an association between the *CYP2B6* c.516C>T polymorphism and an increased risk for cADRs in Black Africans and Caucasians (CICCACCI *et al.*, 2013; YUAN *et al.*, 2011). A strong association between the *CYP2B6* c.983C>T and SJS/TEN was recently described in a Mozambican population (CICCACCI *et al.*, 2013). Hepatotoxicity however, has been associated with a SNP in the *ABCB1* transporter (c.3435C>T) in populations originating in the USA, South Africa and Mozambique (CICCACCI *et al.*, 2010; HAAS *et al.*, 2006; RITCHIE *et al.*, 2006). Additionally, a genome wide association study (GWAS) of NVP-induced rash (NIR) in Thai patients has shown the association of two synonymous SNPs that are in complete LD with a nonsynonymous polymorphism of the *CCHCR1* gene. CCHCR1 has been implicated in the proliferation of keratinocytes increase susceptibility to psoriasis (ASUMALAHTI *et al.*, 2002).

The diverse associations reported with NVP hypersensitivity indicate that population-specific differences in genetic, immunological and metabolic pathways may contribute to the pathogenesis of these reactions.

Table 1.8: Genetic polymorphisms associated with nevirapine-induced hypersensitivity

Gene	Adverse reaction	Population	P -value	Reference
CYP2B6 c.516T>G (rs3745274)	cADRs	Blacks	0.003	(YUAN *et al.*, 2011)
		Whites	0.021	
	SJS/TEN	Mozambicans	0.001	(CICCACCI *et al.*, 2013)
CYP2B6 c.983C>T (rs28399499)	SJS/TEN	Mozambicans	0.026	(CICCACCI *et al.*, 2013)
ABCB1 c.3435C>T (rs1045642)	Hepatotoxicity	Mixed	0.040	(RITCHIE *et al.*, 2006)
		South Africans	0.016	(HAAS *et al.*, 2006)
		Mozambicans	0.038	(CICCACCI *et al.*, 2010)
CCHCR1 (rs126511 and rs746647)	Skin rash	Thai	1.2×10^{-8}	(CHANTARANGSU *et al.*, 2011)

Adverse events as defined by the authors. *cADR* cutaneous adverse drug reaction, *CYP* cytochrome P450 enzyme, *HLA* human leukocyte antigen, *SJS* Stevens-Johnson syndrome, *TEN* toxic epidermal necrolysis

1.6 Aims of this study

Malawi has one of the highest prevalence rates of HIV/AIDS in the world [11% compared to 0.2% and 0.6% in the UK and USA respectively (CENTRAL INTELLIGENCE AGENCY, 2009)]. Currently, over one million people are HIV infected and AIDS is the leading cause of death amongst the 15-49 year olds (UNAIDS, 2012a). Although more beneficial ART regimens are commonly prescribed in developed countries, NVP is widely used as a favourable first-line regimen and the maternal as well as infant MTCT coverage in resource-limited settings like Malawi (DEPARTMENT FOR HIV AND AIDS, 2012).

The number of pharmacogenetics studies performed in African populations is rather limited and only very little information is available from Malawi. African populations feature high levels of genetic diversity and data included in public initiatives, such as the 1000 Genome Project and the International HapMap Project, do not address the ethnically and geographically different African groups (GENOMES PROJECT CONSORTIUM *et al.*, 2012; INTERNATIONAL HAPMAP, 2003). This data suggests that pharmacogenetics data acquired in other African populations may not be easily applied to Malawi.

The inconsistency among the genetic associations observed in NVP hypersensitivity highlight the need for further research to identify and characterise molecular aspects of NVP-induced HSRs. The aims of this project were therefore:

(a) to assess gene expression changes observed in our Malawian population and further characterise these findings in NVP patients as well as healthy volunteers;
(b) to investigate the role of miR-148a and post-transcriptional regulation of HLA-C*04 expression in NVP-hypersensitive patients;
(c) to identify blood-based miRNAs and their potential utility as clinical markers of NVP-induced cutaneous ADRs;
(d) and to review and evaluate the relationship between NVP hypersensitivity and HLA-alleles across different populations including the effect of population specific differences in allele frequency.

Chapter 2

Validation of differential expressed genes and *in vitro* analysis of neutrophil antigen CD177

Contents

Figures

Tables

2.1 Introduction

To identify differentially expressed gene transcripts, gene expression profiling was performed in a black African population of NVP-treated HIV patients from Malawi (manuscript in preparation). Complementary DNA (cDNA) microarray analysis was performed on the Affymetrix GeneChip array and differential gene expression analysis was carried out in blood samples collected from NVP-hypersensitive and tolerant patients. Samples taken from NVP-hypersensitive patients were either classified as acute (taken during the acute phase of the reaction; n = 6) or recovered samples (obtained after drug withdrawal and complete resolution of clinical symptoms; n = 6). In addition six samples from NVP-tolerant were also included in the study. Due to the small number of pre-treatment blood samples available, recovered samples were chosen as the control group. Statistically significant differences in gene expression levels were analysed in contrasts of the acute versus recovered (A vs. R) and tolerant versus recovered (T vs. R) patient groups.

Differential gene expression analysis revealed a total of 225 transcripts to be significantly deregulated in NVP-treated patients. The transcripts with the highest fold increases in expression were homologous to *CD177*, a neutrophil specific antigen, and peptidoglycan recognition protein 1 (*PGLYRP1*), known to bind murein peptidoglycans of gram-positive bacteria. The expression of the inflammation-associated genes *CD177* and *PGLYRP1*, which are now known as endogenous damage-associated patterns (DAMPs), has been shown to be significantly upregulated in severe skin reactions [SJS and TEN (BELLON *et al.*, 2010)].

Pathway analysis of these results showed granulocyte colony-stimulating factor 3 (*CSF3*) to be a central partner of the CD177 protein pathway, which controls the production, differentiation and function of granulocytes (figure 2.1).

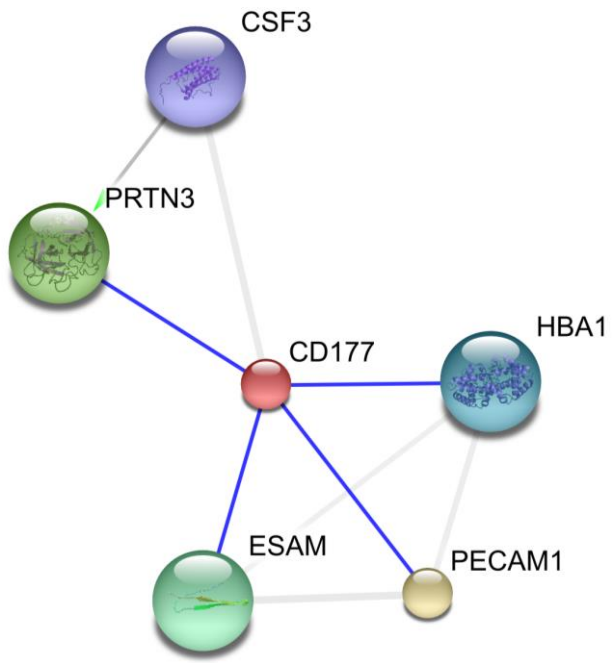


Fig. 2.1: Predicted protein interactions illustrating direct and indirect associations with CD177

Network generated using the Search Tool for the Retrieval of INteracting Genes database [STRING version 8.2 (SZKLARCZYK *et al.*, 2011)]. Differentially expressed proteins are shown as small circles while other proteins are presented as spheres. Blue lines indicate binding properties. *CSF3* granulocyte colony-stimulating factor, *ESAM* endothelial cell-selective adhesion molecule, *HBA1* haemoglobin subunit alpha, *PECAM-1* platelet endothelial cell adhesion molecule, *PRTN3* proteinase 3

Recent findings indicate that the pathogenesis of NVP-induced HSRs is due to a T cell dependent immune response. This is consistent with the associations of different HLA-alleles and NVP hypersensitivity that have been described (summarised in table 1.8). Furthermore higher CD4+ T cell counts have been shown to affect the risk of adverse reactions linked to NVP treatment (BOEHRINGER-INGELHEIM, 2012; CARR *et al.*, 2013; TAIWO, 2006). This theory has been further supported by the histopathological findings of immune cell infiltration in lesions in the animal model developed by Shenton *et al.* (2003) as well as in patients with drug-induced MPE and SJS (PICHLER, 2003; WETTER and CAMILLERI, 2010). Several studies have also shown that T cell migration precedes neutrophilic transmigration in drug-induced acute generalised exanthematous pustulosis (KELLER *et al.*, 2005; SCHAERLI *et al.*, 2004).

CD177, also known as NB1, human neutrophil antigen 2a (HNA-2a) and polycythemia rubra vera 1 (PRV-1), is a membrane protein specifically expressed on neutrophils. It was first described in 1971 in several cases of alloimmune neonatal neutropenia (LALEZARI *et al.*, 1971; TEMERINAC *et al.*, 2000). Heterophilic interactions between CD177 and the platelet endothelial cell adhesion molecule-1 (PECAM-1, also known as CD31) have been shown to

promote transendothelial migration of CD177-positive neutrophils (see figure 2.2) after stimulation with N-formyl-methionyl-phenyl-alanine [fMLP (BAYAT *et al.*, 2010; SACHS *et al.*, 2007)]. Expression of the cell surface protein is restricted to a subpopulation of neutrophils, with the mean population size of CD177-positive neutrophils ranging from 45-65% (CARUCCIO *et al.*, 2004; GOLDSCHMEDING *et al.*, 1992; KISSEL *et al.*, 2001; STRONCEK, 2007). It has been shown that the CD177-negative subpopulation lacks membrane expression due to incomplete mRNA transcription, whereas CD177 deficiency is caused by frame shift mutations leading to the insertion of premature stop codons (KISSEL *et al.*, 2002; WOLFF *et al.*, 2003). CD177-deficient people are healthy, but too few studies have been performed to define if lack of CD177 protein expression has an effect on neutrophil function or the immunological and inflammatory response (JANKOWSKA *et al.*, 2011; WOLFF *et al.*, 2003). Additionally, cell surface expression of proteinase-3 (PR3) has been shown to be dependent on CD177 (BAUER *et al.*, 2007; VON VIETINGHOFF *et al.*, 2007).

Given these data and the discovery of a NVP-dependent increase in *CD177* expression, we hypothesised that neutrophils may contribute to the development and maintenance of NVP hypersensitivity. To determine whether the NVP-dependent increase in *CD177* expression plays a role in the pathophysiology of NVP hypersensitivity, gene and protein expression profiles of patients as well as healthy, NVP-naïve controls were analysed. The specific aims were to (i) validate the results of the *CD177* and *PGLYRP1* microarray analysis but also to investigate the gene expression levels of *CSF3* in two sets of patient samples from Malawi and (ii) further characterise *CD177* gene and protein expression after treatment with NVP *in vitro*.

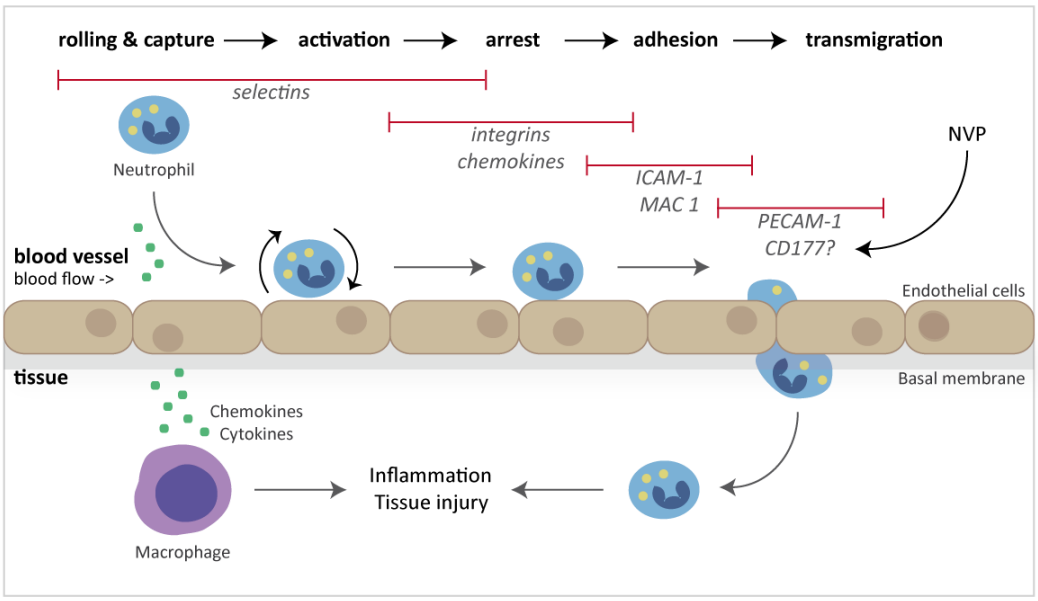


Fig. 2.2: Schematic diagram depicting the neutrophil recruitment cascade
Shown are the steps of paracellular neutrophil recruitment from the vasculature to the tissue. Selectins regulate the rolling, whereas arrest and adhesion depend primarily upon integrin interactions. Adhesion and transmigration is largely controlled by ICAM-1 and PECAM-1. NVP may stimulate CD177 expression thus facilitating the preferential transmigration of CD177-positive cells through heterophilic interactions with PECAM-1 (adapted from: BORREGAARD, 2010; WOODFIN *et al.*, 2009). *ICAM-1* intracellular adhesion molecule 1, *NVP* nevirapine, *PECAM-1* platelet endothelial cell adhesion molecule

2.2 Methods

2.2.1 Patients and data collection

Malawi cohort

Between 2007 and 2009, 1117 HIV-1 positive antiretroviral-naïve patients were recruited prospectively at the Queen Elizabeth Central Hospital in Blantyre, Malawi for a nested case-control study (conducted by Dr. Mas Chaponda). Written informed consent was obtained from patients suitable to commence ART (WHO clinical stage 3/4 or CD4+ T cell count below 250 cells/µl). All patients recruited were self-reported black Africans, 16 years or older and started on Triomune-40, a fixed-dose combination of stavudine (40 mg), lamivudine (150 mg) and NVP (200 mg). Patients were followed up for 26 weeks, with clinical and laboratory parameters such as CD4+ T cell count and liver function tests monitored at 0, 6, 14 and 18 weeks, if possible. Due to the

low incidence (5%) of NVP-induced hypersensitivity, an additional 177 patients, who developed NVP hypersensitivity and attended the same outpatient clinic, were recruited into the study either as patients presenting de novo in clinic (n = 149) or identified from patient records retrospectively (n = 28) during the same time period. Ethics committees at the Liverpool School of Tropical Medicine and the College of Medicine, Malawi, approved the study. Data on ethnicity, gender, age, weight, height as well as CD4+ T cell count, treatment duration and HSR were collected from all patients. The identification and characterisation of HSRs was performed using the Naranjo Probability Scale [summarised in table 2.1 (NARANJO *et al.*, 1981)]. In accordance with international guidelines, patients with any kind of drug-induced HSRs described below had NVP withdrawn.

Using both clinical data and photographs, phenotypes were retrospectively reviewed by a dermatologist blinded to the initial classification of cases. Patients who presented one of the following hypersensitivity reactions were classified as NVP-hypersensitive patients (CARR *et al.*, 2013):

(i) NIR: widespread maculopapular rash without systemic manifestations that deteriorates with treatment continuation;
(ii) HSS: widespread rash with systemic manifestations like fever, cough or abnormal liver function tests;
(iii) Extensive rash with involvement of at least 2 mucous membranes or skin lesions affecting less than 10% (SJS) or more than 30% (TEN) of the body surface area (FAGOT *et al.*, 2001). Blistering between 10% and 30% of body surface area was labelled SJS/TEN overlap syndrome;
(iv) DILI: visible jaundice and/or elevated alanine aminotransferase levels.

Although patients who were treated for concomitant tuberculosis or hepatotoxicity, were currently pregnant or had previously received ART were excluded from the study, potential confounding through incomplete clinical information and indigenous herbalism cannot be excluded. The control group consisted of patients treated with NVP for at least six months without the occurrence of any type of hypersensitivity.

Table 2.1: Summary of the Naranjo adverse drug reaction probability scale

Question	Yes	No	Do not know
Are there previous conclusive reports on this reaction?	+1	0	0
Did the adverse event occur after the suspected drug was administered?	+2	-1	0
Did the adverse reaction improve when the drug was discontinued or a specific antagonist was administered?	+1	0	0
Did the adverse reaction reappear when the drug was re-administered?	+2	-1	0
Are there alternative causes (other than the drug) that could have on their own caused the reaction?	-1	+2	0
Did the reaction reappear when a placebo was given?	-1	+1	0
Was the drug detected in the blood (or other fluids) in concentrations known to be toxic?	+1	0	0
Was the reaction more severe when the dose was increased or less severe when the dose was decreased?	+1	0	0
Did the patient have a similar reaction to the same or similar drugs in any previous exposure?	+1	0	0
Was the adverse event confirmed by any objective evidence?	+1	0	0

The Naranjo criteria summarised above classify the probability that an ADR is related to a specific drug. ADRs are assigned to a probability category based on the overall score: **definite ADR** for a score ≥ 9, **probable ADR** if the score is 5 to 8, **possible ADR** for 1 to 4 and **doubtful ADR** if the score is 0 (adapted from: NARANJO *et al.*, 1981). *ADR* adverse drug reaction

A subset of 47 NVP-treated patients were randomly chosen from the above cohort for further analysis (see figure 2.3 or table 2.2). Of these patients, 30 (21 NVP-hypersensitive and 9 tolerant controls) had 36 *in vivo* samples available for the gene expression analysis: samples from 17 hypersensitive patients were taken during the HSR (acute); after drug withdrawal and complete resolution of clinical symptoms, a recovered sample was obtained from ten NVP-hypersensitive patients, while samples from nine tolerant patients were also included in the study.

The *in vitro* analysis comprised 52 samples taken from 17 hypersensitive and 16 tolerant patients that were subsequently treated with NVP or dimethyl sulfoxide (DMSO; see section 2.2.2). For the statistical analysis of the *in vitro* data, only patients with both DMSO and NVP-treated samples were included in the study as fold change ($2^{-\Delta\Delta C_T}$) can only be calculated from paired samples (section 2.2.5). Thus *CD177, PGLYRP1* and *CSF3* expression in nine hypersensitive patients and nine tolerant controls were analysed.

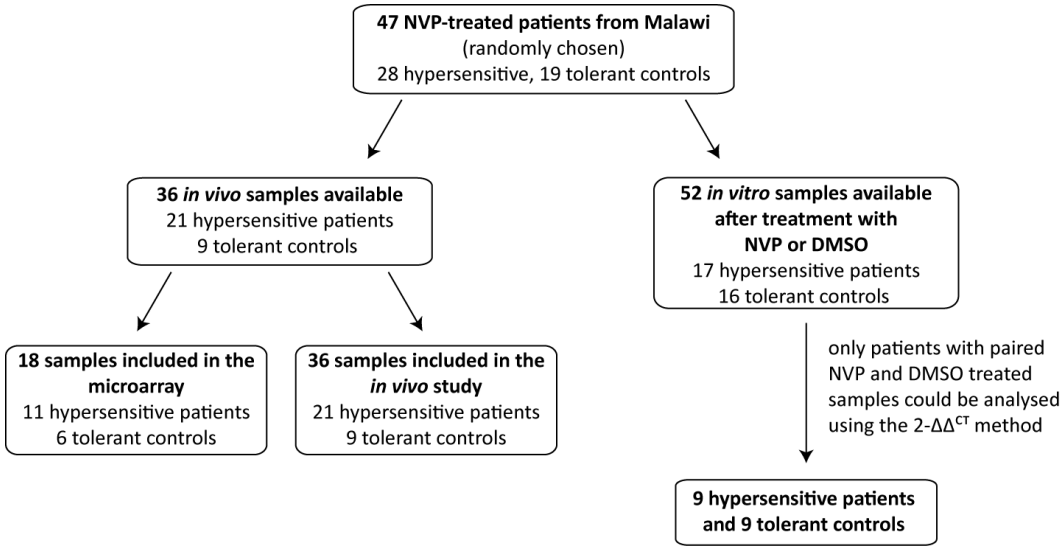


Fig. 2.3: Flowchart depicting the various subsets of Malawian patients that were used in this study

If more samples were available than indicated patients, paired or duplicated samples were used.

Table 2.2: Characteristics of Malawian patients

ID	Phenotype	Gender	Age	Weight	Height	CD4	Samples				
							in vivo			*in vitro*	
							A	R	T	DMSO	NVP
NR0142	DILI	Female	31	42	1.41	321	x*	x*			x†
NR0152	DILI	Male	28	43	1.62	58	x*				
NR0154	DILI	Female	32	42.2	1.64	248	x*				
NH1109	HSS	Female	32	52.2	1.6	145	x				
NR0034	HSS	Male	26	56	1.8	NA					x†
NR0134	HSS	Male	39	52.6	1.64	191				x	x
NR0140	HSS	Female	43	44	1.51	656				x	x
NR0141	HSS	Female	37	57.1	1.55	235	x	x*		x[a]	x[a]
NH0516	NIR	Male	35	61.8	1.8	98					x†
NH0834	NIR	Male	41	66	1.63	122		x *		x	x
NH1035	NIR	Male	35	69.9	1.82	220	x				
NH1041	NIR	Female	43	47.2	1.5	70	x			x†	
NH1060	NIR	Female	33	54.1	1.4	332	x				
NR0092	NIR	Female	35	56.4	1.57	57		x*			
NR0145	NIR	Female	38	46.1	1.47	173	x				
NR0146	NIR	Female	26	58.1	1.62	286	x	x*		x	x
NR0155	NIR	Female	38	56.9	1.68	234	x*				
NH0821	SJS	Female	43	65	1.71	167		x		x	x
NH0993	SJS	Female	34	48.6	1.61	168		x			x†
NR0049	SJS	Female	27	51	1.6	NA					x†
NR0135	SJS	Male	37	60.7	1.73	574				x	x
NR0136	SJS	Female	28	56.4	1.62	337				x	x
NR0137	SJS	Female	19	54	1.59	161	x				
NR0138	SJS	Male	58	57	1.59	522	x*				
NR0139	SJS	Female	37	49.2	1.49	172	x*	x		x†	
NR0143	SJS	Female	54	35.8	1.49	105	x	x		x	x
NR0144	SJS	Female	23	53	1.72	357	x	x*		x	x
NR0153	SJS	Male	35	63.6	1.66	371	x				
NH0917	Tolerant	Male	37	54.5	1.65	133			x		
NH0957	Tolerant	Female	40	66	1.61	7				x†	
NH0959	Tolerant	Female	27	46.1	1.56	306			x*	x	x
NH0966	Tolerant	Male	60	71.1	1.7	43			x*		
NH0967	Tolerant	Female	44	79.8	1.49	20			x*	x	x
NH0968	Tolerant	Male	55	54.7	1.59	58			x*	x	x
NH0970	Tolerant	Male	39	56	1.67	91			x	x†	
NH0973	Tolerant	Female	22	53.5	1.6	105			x*	x	x
NH0974	Tolerant	Female	29	56.7	1.65	192			x	x	x
NH0975	Tolerant	Female	40	52.4	1.66	263					x†
NH0980	Tolerant	Female	56	47.1	1.48	117			x*		
NH0983	Tolerant	Male	35	50.9	1.6	130				x†	
NH1021	Tolerant	Female	29	48.3	1.63	54					x †

Table 2.2: continued

ID	Phenotype	Gender	Age	Weight	Height	CD4	Samples				
							in vivo			*in vitro*	
							A	R	T	DMSO	NVP
NH1022	Tolerant	Male	35	74.2	1.76	165				x	x
NH1023	Tolerant	Male	32	48.6	1.64	284				x	x
NH1024	Tolerant	Female	41	46.5	1.59	232				x	x
NH1025	Tolerant	Male	42	65.4	1.59	242					x†
NH1026	Tolerant	Female	26	57.2	1.58	74					x†
NH1029	Tolerant	Male	53	64.5	1.66	34				x	x

A total of 47 patients were randomly chosen for this study. The *in vivo* samples taken from patients is indicated as A (acute), R (recovered) and T (tolerant). Samples treated with either DMSO or NVP are also listed. (*) denotes samples included in the microarray; (†) marks samples which were excluded from the final analysis as calculation of $2^{-\Delta\Delta C}{}_{T}$ was not possible; (a) could not be amplified. *CD4* CD4+ T cell count, *DILI* drug-induced liver injury, *HSS* hypersensitivity syndrome, *NIR* nevirapine induced rash, *NVP* nevirapine, *SJS* Stevens-Johnson Syndrome

Patients and volunteers recruited in Liverpool

Six HIV-1 positive, NVP-hypersensitive patients and seven healthy controls were recruited at the University of Liverpool and the Liverpool and Broadgreen University Hospital in 2011 and 2012. Subjects were of self-reported Caucasian or black African ancestry. Ethical approval was granted by the North West Centre Research Ethics Committee (UK) and written informed consent was obtained from all subjects. Healthy controls were recruited prospectively, whereas NVP-hypersensitive patients were recruited retrospectively. Five of the six hypersensitive patients developed NVP-induced HSRs (as classified above) one to nine years before samples were taken and were no longer on NVP containing ART; one patient was recruited during the acute phase of the reaction and samples were taken two days after NVP was withdrawn.

2.2.2 *In vitro* culture of whole blood

This part of the study was carried out by Dr. Mas Chaponda for samples collected in Malawi and by myself for samples collected in the UK.

Whole blood collected in lithium heparin Vacuette® Blood collection tubes (Greiner Bio-One, Germany) was diluted with four volumes of RPMI 1640 media (Sigma Aldrich, USA), containing 100 U/ml penicillin and 100 µg/ml

streptomycin. Diluted whole blood was then treated with 25 µM and 50 µM NVP (Sigma Aldrich, USA), which lie within or slightly above the therapeutic range observed in NVP-treated individuals (11–30 µM). In addition, samples collected in Liverpool were treated with 20 µM fMLP (Sigma Aldrich, USA), a chemoattractant that was included as a positive activation control.

Studies have shown that 30 min of fMLP treatment is sufficient to induce CD177 protein expression and reproduce inflammatory changes of neutrophils observed *in vivo* (SACHS *et al.*, 2007; VIDEM and STRAND, 2004). In addition, preliminary flow cytometry analysis showed that the percentage of granulocytes in a heterogeneous leukocyte population decreases from approximately 40% to 10% after 24 hours at standard cell culture conditions (37°C, 5% CO_2; data not shown). For this reason, samples collected in Liverpool were incubated for either 30 min or three hours to account for possible time dependent changes in gene and protein expression. However, the only available RNA samples collected in Malawi had been incubated for 24 hours. All samples were incubated under standard cell culture conditions before mRNA and protein expression were analysed as described below.

Nevirapine and fMLP were diluted in a 10% DMSO solution to a final concentration of 0.01 M. A 10% DMSO solution was used as vehicle control.

2.2.3 RNA extraction

PAXgene Blood RNA extraction of Malawi patient samples

Performed by Dr. Mas Chaponda.

PAXgene Blood RNA tubes (PreAnalytix, QIAGEN, Germany) were used for the collection of whole blood in Malawi. Total RNA extraction was performed on peripheral blood mononuclear cells (PBMCs) using the PAXgene™ Blood RNA kit (PreAnalytix, QIAGEN, Germany), followed by the reduction of alpha- and beta-globin mRNA using the GLOBINclear™ kit (Ambion, Applied Biosystems, USA) according to the manufacturer's protocol.

E.Z.N.A. RNA extraction of cultured whole blood

Using the E.Z.N.A.® RNA isolation kit (Omega Bio-Tek, USA), total RNA was extracted from cultured whole blood samples as stated by the manufacturer. All samples were eluted in 50 µl diethylpyrocarbonate-treated (DEPC) water. Samples were concentrated to a final volume of 20 µl using the Vacufuge® vacuum concentrator (Eppendorf, Germany).

Total RNA quality and quantity was determined by spectro-photometry (NanoDrop 8000, Thermo Fischer Scientific, USA). Integrity of the RNA samples collected in Malawi was analysed using the Agilent 2100 Bioanalyzer and the Agilent RNA 6000 Nano kit (Agilent Technologies, USA). Samples with a RNA integrity number higher than five and a 260/280 mm ratio of absorbance higher than 1.6 were included in these experiments. All RNA samples were stored at -80°C.

2.2.4 Probe based real-time polymerase chain reaction

Reverse transcription

RNA samples were thawed on ice and up to 260 ng of total RNA was reverse transcribed to cDNA with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) according to the manufacturer's protocol. Twenty microliter reactions containing 2X reverse transcriptase buffer, 0.25 mM dNTP mix, 2 µl random primers, 1 µl RNase inhibitor, 50 U reverse transcriptase and 260 ng RNA was added to a 96-well plate. Reverse transcription was performed on the Veriti® Thermal Cycler (Applied Biosystems, USA) as follows: 10 min at 25°C, 120 min at 37°C and 5 min at 85°C.

Real-time qPCR reaction

TaqMan® Gene Expression assay technology utilizes sequence-specific oligonucleotide probes and primers. Fluorophores, such as 6-carboxy-fluorescein (6FAM™; simply known as FAM), and quencher molecules are hybridized to the 5' end and the 3' end of the probe, respectively. While the

probe is bound to a single-stranded DNA molecule, the fluorophore is located in close proximity to the quencher, which inhibits the fluorescence signal. Extension of the unlabelled primers on the template cDNA strand by the Taq polymerase leads to degradation of the probe. This releases the fluorophore and leads to an increase in fluorescence intensity, which is proportional to the amount of cDNA molecules present.

A 20 µl reaction containing 10 µl gene expression master mix, 1 µl gene expression assay (table 2.3), 5.9 µl water and 3.1 µl cDNA was added to a 384-well plate. All samples were run in duplicate and no template controls (NTCs) were included as part of quality control on a 384-well plate. Thermal cycling was performed on the Applied Biosystems HT 7900 Fast Real-Time PCR System (Applied Biosystems, USA) under the following conditions: 10 min at 95°C followed by 45 cycles of a denaturing step at 95°C for 15 seconds and an annealing step at 60°C for 1 min.

Table 2.3: TaqMan® Gene expression Assays

Gene ID	Gene name	Fluorophore/Quencher	Assay ID
ACTB	Beta-actin	FAM/NFQ	Hs99999903_m1
CD177	CD177 molecule	FAM/NFQ	Hs00360671_m1
CSF3	colony stimulating factor 3 (granulocyte)	FAM/NFQ	Hs99999083_m1
PGLYRP1	peptidoglycan recognition protein 1	FAM/NFQ	Hs00175475_m1

Supplied by Applied Biosystems (USA). *FAM* 6-carboxy-fluorescein, *NFQ* nonfluorescent quencher

2.2.5 Gene expression analysis

Gene expression is quantified by the number of cycles at which the amplification curve crosses the threshold value, i.e. the fluorescence signal surpasses background signal (C_T); these were determined using the SDS software (version 2.2; Applied Biosystems, USA).

Gene expression analysis of Malawian in vivo patient samples

Using the comparative C_T method, the difference between the mean target C_T value (gene of interest) and the mean control C_T value (reference gene; beta-actin) was calculated for each sample.

The level of expression was then determined using the ΔCT method (LIVAK and SCHMITTGEN, 2001): $\Delta C_T = C_T\ (gene\ of\ interest) - C_T(refrence\ gene)$

The expression was calculated as individual data points using $2^{-\Delta C_T}$. Difference in gene expression is shown as fold change:

$$2^{-\Delta C_T}(hypersensitive\ patients) \Big/ 2^{-\Delta C_T}(tolerant\ patients)$$

Analysis of gene expression changes observed in cultured whole blood

To compare the gene expression of treated and untreated isolated leukocytes, the mean for both the gene of interest and reference gene were calculated and the fold change = $2^{-\Delta\Delta C_T}$ determined. This is achieved by calculating the ΔΔCT as follows with beta-actin as reference gene:

$$\{(C_T\ gene\ of\ interest - C_T\ reference\ gene)_{NVP_{treated}} - (C_T gene\ of\ interest - C_T reference\ gene)_{DMSO_{treated}}\}$$

2.2.6 Flow cytometry analysis of surface antigen expression

Cell surface antigen expression [recorded as the mean fluorescence intensity (MFI)] and percentage of antigen presenting cells were determined by labelling leukocytes with a FITC conjugated, CD177 antigen specific antibody [mouse anti-human (clone MEM-166) available at abcam®, UK]. A 500 µl aliquot of cultured whole blood (see 2.2.2) treated with either DMSO, NVP (25 or 50 µM) or fMLP (20 µM) was pelleted at 500 x g for 5 min at room temperature and fixed in 500 µl 1x CellFIX (BD™ Biosciences, USA) for 20 min on ice. After centrifugation, red blood cells were lysed by addition of 1.2 ml 1x FACS Lysing Solution (BD™ Biosciences, USA) and incubated for 20 min at room

temperature. Cells were then washed by addition of 1.2 ml Hanks' Balanced Salt solution (HBSS; Sigma Aldrich, USA) and subsequent centrifugation at 500 x g for 5 min at room temperature; this was repeated twice. The cell pellet was resuspended in 200 µl HBSS and incubated with 20 µl of FITC-labelled CD177 antibody for up to one hour in the dark and on ice. To remove excess unbound antibody, cells were washed twice as previously described. Following the last centrifugation step, the pellet was resuspended in 500 µl HBSS and the fluorescent signals acquired on the FACS Canto II system using the FACSDiva software (version 6.1.2; BD™ Biosciences, USA). Events were collected twice for each sample. Data were analysed using FlowJo version 8.7 (TREE STAR INC., 2013). Representative results of granulocyte specific gating and histograms are shown in figure 2.4. By placing the second gate at the valley of the positive and negative peaks, CD177-positive and CD177-negative populations were determined.

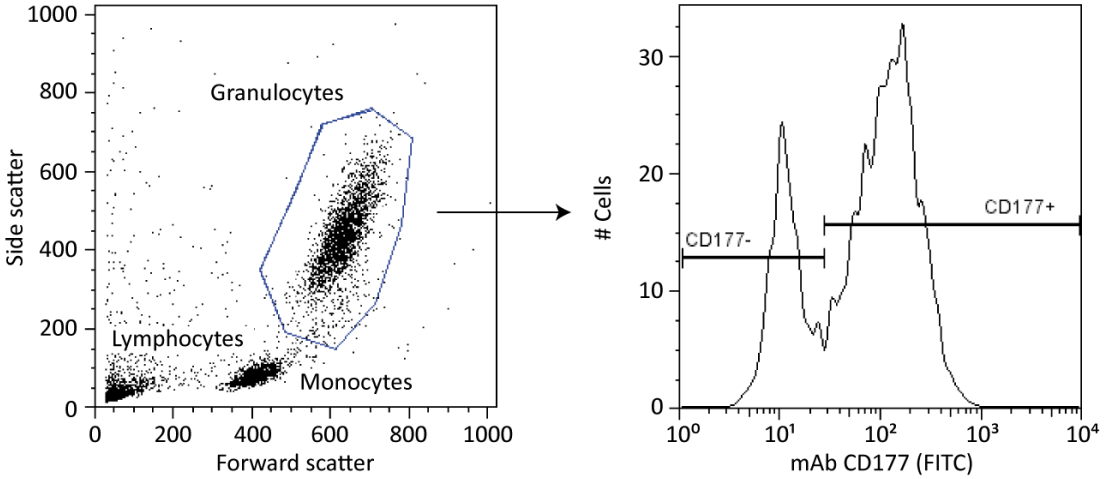


Fig. 2.4: Gating strategy for analysis of CD177 protein expression on granulocytes
Leukocytes are first gated on a forward scatter/side scatter dot plot. The granulocytes are then visualised using a CD177 histogram in which CD177-negative and CD177-positive cells are calibrated by placing the second gate at the valley between negative and positive peaks; CD177 positive cells appear in the CD177+ gate.

2.2.7 Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics version 20 (IBM Corp., USA) or the genetics package of R (R CORE TEAM, 2013).

As many of the analyses performed in this thesis required the application of more than one statistical test, p values were adjusted using multiple testing corrections in order to correct for the occurrence of false-positive results. The Bonferroni correction method is the simplest and most extensively used method for small numbers of comparisons in which the p value is divided by the number of statistical tests performed. The False Discovery Rate (FDR) represents a less stringent approach and is generally used to control for larger numbers of comparisons (NOBLE, 2009). Although the approach to correct for multiple testing can be extremely conservative in *in* vitro experiments, these methods increase the power of identifying true associations within our studies.

Analysis of observed gene expression changes

To ensure normal distribution of the data, CD4+ T cell count was transformed using the natural logarithm ($\log_e$; corresponding histograms are shown in appendix 2.1).

Gene expression data of patient samples collected in Malawi were compared using a multilevel linear model to account for the fact that repeated measurements were taken from some but not all patients (paired acute and recovered samples). Results were adjusted for multiple testing using the Bonferroni correction method; a Bonferroni threshold of $p = 0.05/4 = 0.0125$ [two tests per gene (A vs. R and T vs. R), two genes in total tested] indicates statistically significant results.

Changes in gene expression levels observed between treated and untreated leukocytes from patients and volunteers were analysed using an independent sample or paired sample t-tests. The independent sample t-test was chosen to analyse the difference in fold change between hypersensitive and tolerant patients or healthy volunteers, whereas the paired sample t-test was used to test for inter-individual differences of control versus treatment. The FDR approach was used to control for the multiple tests used on *in vitro* treated samples from Liverpool.

Flow cytometry

Differences in surface antigen expression (MFI) and percentage of antigen presenting cells were analysed using a paired sample t-test. Data were log_{10} transformed to ensure normal distribution (see appendix 2.2). The FDR was calculated for each test due to the large number of comparisons made.

2.3 Results

2.3.1 Patient and volunteer demographics

Characteristics of the 47 patients recruited in Malawi are summarised in table 2.4. Twenty of the 28 hypersensitive patients were recruited as part of the retrospective study. The majority of tolerant and hypersensitive patients were female (53% and 68% respectively). Mean body weight and CD4+ T cell count were 54 kg and > 200 cells/μl in hypersensitive patients, whereas tolerant controls had a higher mean body weight of 58 kg but lower CD4+ T cell count (< 150 cells/μl).

Eight (38%) of the 21 hypersensitive patients included in the *in vivo* analysis experienced NIR, while two (10%) developed HSS and eight (38%) SJS. Three (14%) experienced DILI. Of the nine hypersensitive patients included in the *in vitro* analysis, five (56%) developed SJS, two (22%) HSS, and two (22%) NIR.

Table 2.4: Demographic data of NVP-treated patients from Malawi

	Tolerant (n = 19)	Hypersensitive (n = 28)
Age, median years (IQR)		
	39 (29 – 44)	35 (29 – 39)
Gender, n (%)		
Male	9 (47)	9 (32)
Female	10 (53)	19 (68)
Body weight, kg (range)		
	57.6 (48.6 – 65.4)	53.6 (47.5 – 57.9)
CD4+ T cell count (at NVP initiation), mean cells/μl (range)		
	134 (54 – 232)	228 (109 – 329)

Table 2.4: continued

	Tolerant (n = 19)	Hypersensitive (n = 28)
Hypersensitivity reaction, n (%)		
NIR	-	9 (32)
HSS	-	5 (18)
SJS	-	11 (39)
DILI	-	3 (11)

DILI drug-induced liver injury, *HSS* hypersensitivity syndrome, *IQR* interquartile range, *NIR* nevirapine-induced rash, *SJS* Stevens-Johnson syndrome

The clinical and demographic data of patients and healthy controls that were recruited in Liverpool are summarised in table 2.5.

Four of the patients were female black Africans; three experienced DILI and one developed SJS. One sample (NHL015) failed gene expression analysis. The remaining two patients were male Caucasians who experienced DILI and NIR. Of the seven healthy volunteers four were male; three reported to be of black African origin

Table 2.5: Clinical data of former NVP-hypersensitive patients and healthy volunteers recruited in the UK

	Healthy volunteers (n = 7)	Hypersensitive patients (n = 6)
Age, median years (IQR)		
	30 (28 – 34)	45 (39 – 49)[a]
Gender, n (%)		
Male	4 (57)	2 (33)
Female	3 (43)	4 (67)
Ethnicity		
Caucasian	4 (57)	2 (33)
Black African	3 (43)	4 (67)
Hypersensitivity reaction, n (%)		
NIR	-	1 (17)
HSS	-	-
SJS	-	1 (17)
DILI	-	4 (66)

[a] Age missing for one patient. *DILI* drug –induce liver injury, *HSS* hypersensitivity syndrome, *IQR* interquartile range, *NIR* nevirapine-induced rash, *SJS* Stevens-Johnson syndrome

2.3.2 Validation of microarray results in patient samples from Malawi

Gene expression profiles in peripheral blood mononuclear cells of NVP-treated patients recruited in Malawi

Thirty-six available patient samples were used to validate the gene expression levels of *CD177*, *PGLYRP1* and *CSF3* by qPCR analysis. The expression of acute and tolerant samples was compared to the expression observed in recovered samples.

The analysis by qPCR using a multi-level linear regression model showed that *CD177* (figure 2.5 A) and *PGLYRP1* (figure 2.5 B) were both significantly upregulated during the acute phase of NVP-induced HSRs as well as tolerant patients. *CD177* expression was 70 times higher in acute hypersensitive patients ($p = 0.0008$) compared to a 28-fold upregulation in tolerant controls ($p = 0.0001$). In addition, acute hypersensitive patients showed a significant 5-fold increase in *PGLYRP1* gene expression ($p = 0.0001$) whereas in tolerant patients, *PGLYRP1* was 3.5 times higher expressed than in recovered ($p = 0.005$). Results remained statistically significant after Bonferroni correction for multiple testing (significant threshold: $0.05/4 = 0.0125$). Gene expression of *CSF3* could not be analysed, as *CSF3* was only amplified in seven of the 36 samples included in this study.

Gene expression analysis after treatment with nevirapine in vitro

Nine hypersensitive and nine tolerant patients had both DMSO and NVP-treated samples available and were therefore qualified for the analysis using the $2^{-\Delta\Delta C_T}$ method. The amplification of *CSF3* failed for one hypersensitive patient and one tolerant control.

No statistically significant difference in the gene expression levels of *CD177* ($p = 0.178$, figure 2.6 A), *PGLYRP1* ($p = 0.872$, figure 2.6 B) and *CSF3* ($p = 0.671$, figure 2.6 C) between hypersensitive and tolerant patients could be detected after 24 hour treatment with NVP *in vitro*.

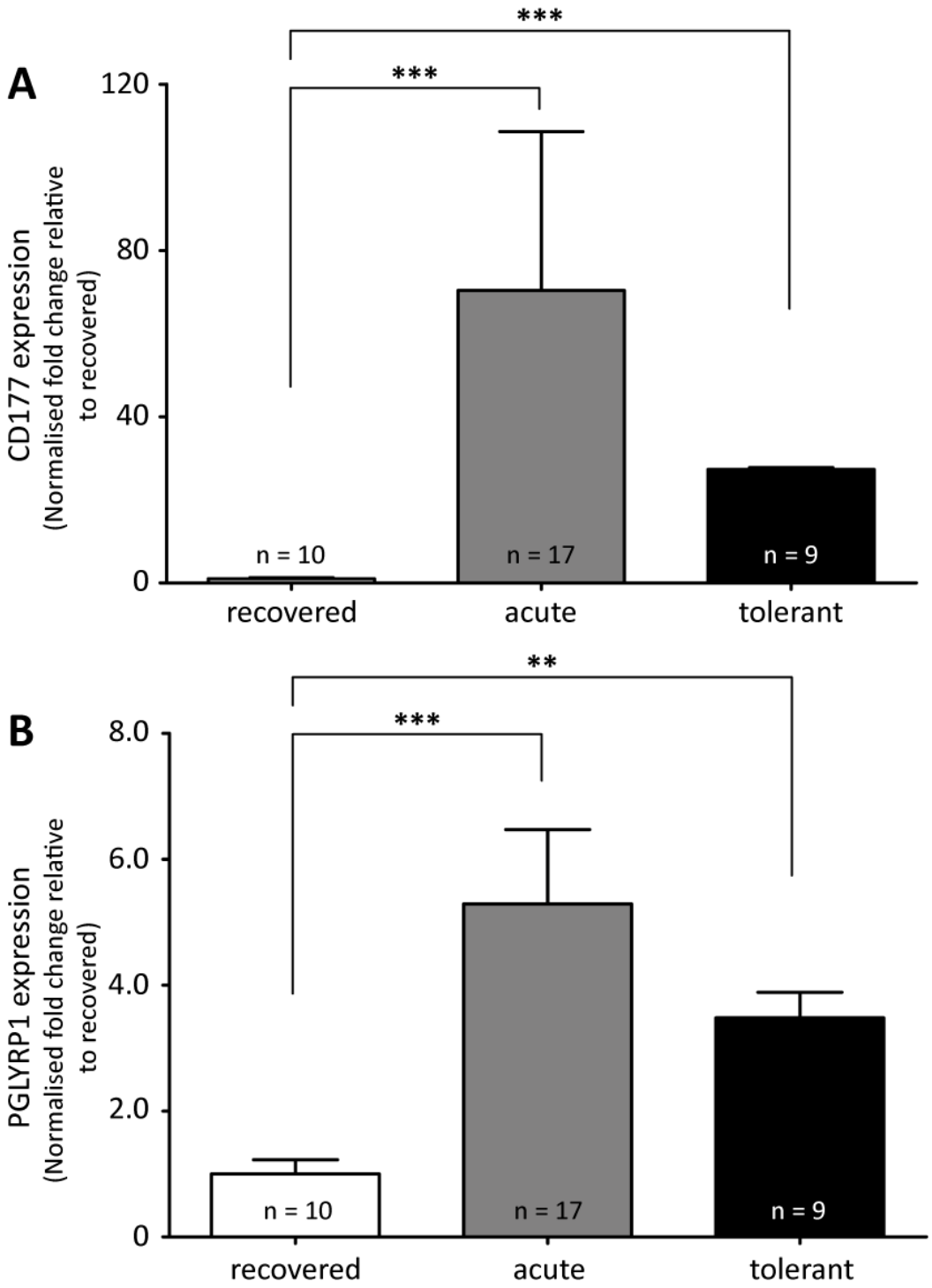


Fig. 2.5: Validation of the microarray array results by qPCR analysis of patient samples
Expression levels of (**A**) *CD177* and (**B**) *PGLYRP1* are analysed by comparison of acute (light grey bars) and tolerant (black bars) patient samples to recovered (white bars). Acute and tolerant patients show a significant upregulation in *CD177* and *PGLYRP1*. Mean gene expression ratio is calculated using the $2^{-\Delta C}{}_{T}$ method and fold change represents the normalised gene expression levels of acute or tolerant patients to recovered. Bars represent fold change ± SEM. Data was analysed using a multilevel linear model and corrected for multiple testing. *PGLYRP1* peptidoglycan recognition protein 1, *SEM* standard error of the mean; ** $p \leq 0.01$ *** $p \leq 0.001$

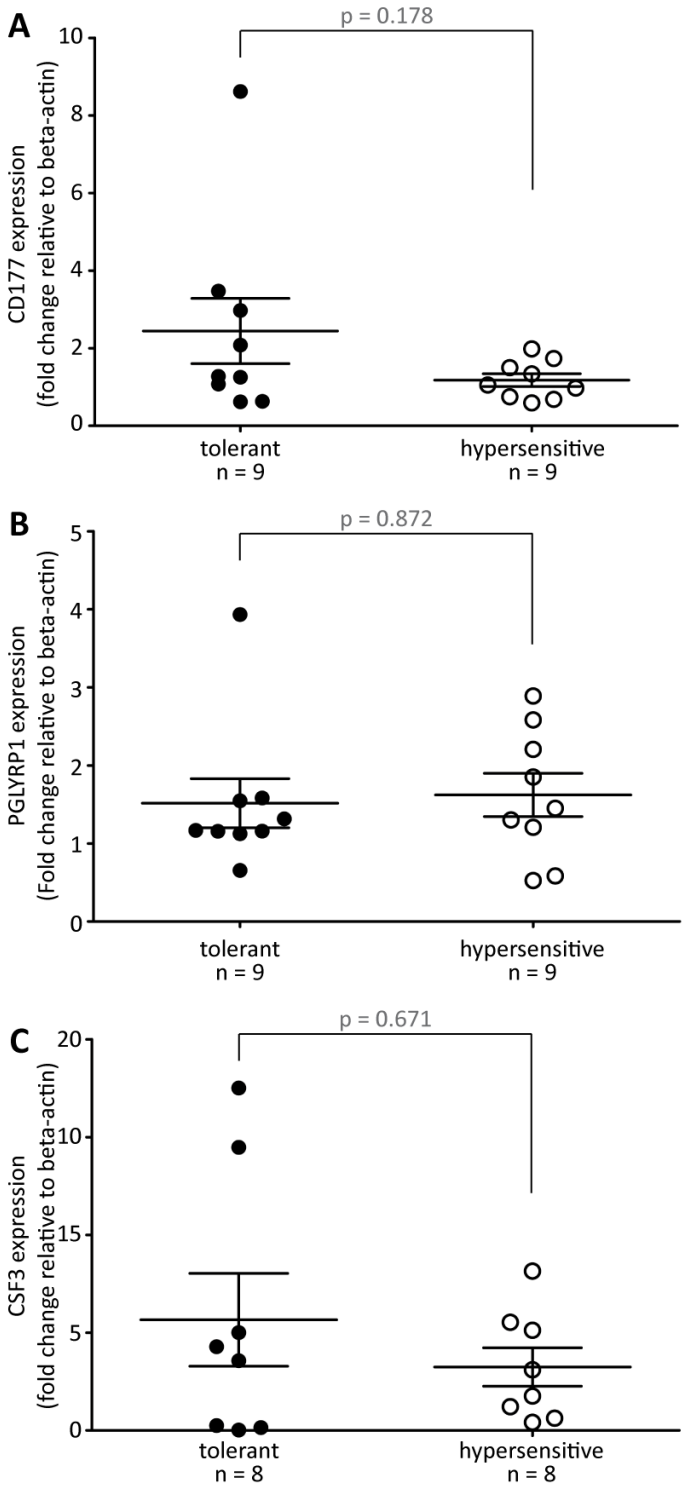


Fig. 2.6: Verification of microarray array results in *in vitro* treated samples from Malawi

In vitro treatment of whole blood samples with NVP or DMSO for 24 hours did not significantly increase the expression of (**A**) *CD177*, (**B**) *PGLYRP1* or (**C**) *CSF3* tolerant (black dots) and hypersensitive patients (white dots). Fold change was calculated and the by $2^{-\Delta\Delta C}{}_{T}$. Lines represent mean ± SEM. Difference between hypersensitive and tolerant patients was compared using an independent sample t-test. *CSF3* colony stimulating factor 3, *DMSO* dimethyl sulfoxide, *PGLYRP1* peptidoglycan recognition protein 1, *SEM* standard error of the mean

2.3.3 CD177 gene expression analysis in patient and volunteer samples from the UK

Based on the results of the microarray and qPCR validation, *CD177* expression was also investigated in an independent cohort of five NVP-hypersensitive

patients and seven healthy, NVP-naïve volunteers after treatment with 25 µM and 50 µM NVP *in vitro*.

First, *CD177* expression was compared between patients and healthy volunteers for each treatment and time point individually. After 30 min a significant difference in *CD177* expression could be detected between patients and healthy volunteers treated with 25 µM ($p < 0.001$) and 50 µM NVP ($p < 0.01$; figure 2.7). In addition a significant difference was found between volunteer and patient samples treated with 25 µM NVP for three hours ($p < 0.05$; figure 2.7), whereas the treatment with 50 µM NVP did not result in a significant increase of CD177 expression levels under the same conditions ($p = 0.10$).

Next the expression of *CD177* was analysed across the patient and volunteer groups respectively. A significant difference in the *CD177* expression was observed between patient samples treated with 25 µM or 50 µM NVP for 30 min ($p = 0.02$), while no difference in the gene expression could be detected after three hours ($p = 0.922$). In healthy volunteers neither treatment for 30 min ($p = 0.843$) nor three hours ($p = 0.966$) lead to a significant difference between samples treated with 25 µM or 50 µM NVP.

However, after correction for multiple testing using the FDR approach only the differences observed between patients and healthy volunteers after treatment with 25 µM and 50 µM for 30 min NVP remained statistically significant (FDR = 0.003 and 0.032 respectively, table 2.6).

Table 2.6: Results of *CD177* expression analysis between former NVP-hypersensitive patients and healthy volunteers

Data analysed using an independent sample t-test	**Volunteers vs. patients**	
	30 min (FDR)	**3 hours (FDR)**
25 µM NVP	**0.0002 (0.0033)**	0.019 (0.067)
50 µM NVP	**0.004 (0.032)**	0.104 (0.235)

Data analysed using a paired sample t-test	**Volunteers**		**Patients**	
	30 min (FDR)	**3 hours (FDR)**	**30 min (FDR)**	**3 hours (FDR)**
25 µM vs. 50 µM NVP	0.843 (0.966)	0.966 (0.966)	0.024 (0.073)	0.922 (0.966)

P values that remained statistically significant after FDR correction for multiple testing are shown in bold. *DMSO* dimethyl sulfoxide, *NVP* nevirapine

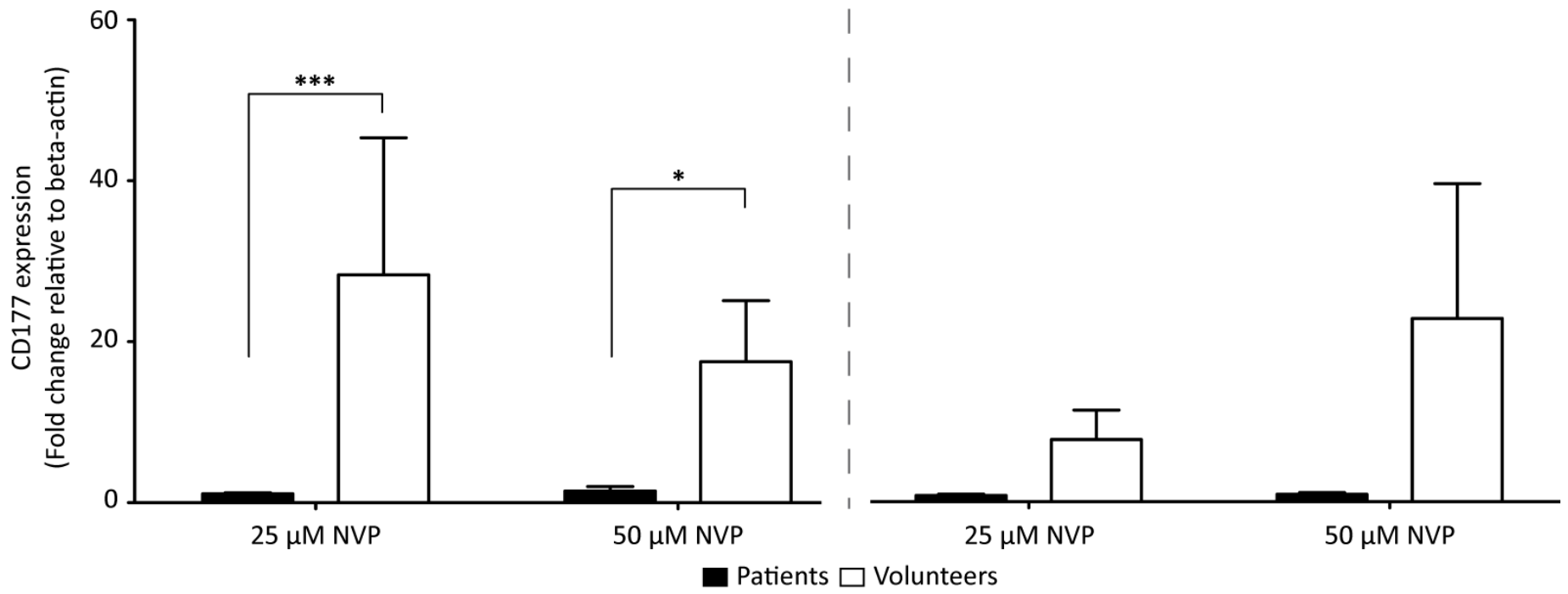


Fig. 2.7: *CD177* gene expression analysis in patients and healthy volunteers from Liverpool

Expression of *CD177* was analysed by qPCR after treatment of whole blood with 25 µM and 50 µM NVP for 30 min (left panel) or 3 hours (right panel). Differences between patients and healthy volunteers were analysed using an independent t-test, whereas inter-group comparisons of treatment versus control were performed using a paired sample t-test. Correction for multiple testing was performed using the FDR approach. *CD177* expression was significantly upregulated in patients treated with 25 µM and 50 µM NVP for 30 min when compared to healthy volunteers. Fold change was calculated using $2^{-\Delta\Delta C}{}_{T}$ after normalisation to beta-actin. Bars represent fold change ± SEM in patients (black bars, n = 5) and healthy controls (n = 7). *NVP* nevirapine, *SEM* standard error of the mean

2.3.4 *In vitro* analysis of CD177 cell surface expression in samples from Liverpool patients

To further characterise the NVP-dependent increase in *CD177* expression, we investigated whether *in vitro* treatment with NVP increases the CD177 surface expression (MFI) as well as the percentage of CD177-positive granulocytes in NVP-hypersensitive patients by flow cytometry. A typical histogram is shown in figure 2.8 (A and B).

Treatment of whole blood with NVP and fMLP for 30 min did not result in an increased expression of CD177 on the cell surface (figure 2.8 C). Likewise, treatment with 25 µM and 50 µM NVP for three hours did not increase the CD177 MFI levels significantly. Only treatment with 20 µM fMLP and co-treatment of fMLP with 25 µM and 50 µM NVP for three hours resulted in a significant increase in CD177 expression ($p < 0.01$). However, after correction for multiple testing only treatment with fMLP alone and co-treatment of fMLP with 50 µM NVP caused a statistically significant increase in protein expression (FDR = 0.049; p values and FDRs are summarised in table 2.7).

Table 2.7: Effects of *in vitro* treatment on the expression and portion of CD177-positive granulocytes

Treatment	MFI		% of CD177+ granulocytes	
(Normalized to DMSO control)	p value	FDR	p value	FDR
30 min				
25 µM NVP	0.506	0.829	0.710	0.840
50 µM NVP	0.309	0.829	0.737	0.840
20 µM fMLP	0.130	0.770	0.396	0.829
20 µM fMLP + 25 µM NVP	0.145	0.770	0.379	0.829
20 µM fMLP + 25 µM NVP	0.154	0.770	0.598	0.829
3 hours				
25 µM NVP	0.267	0.829	0.803	0.845
50 µM NVP	0.422	0.829	0.682	0.840
20 µM fMLP	**0.002**	**0.049**	0.414	0.829
20 µM fMLP + 25 µM NVP	0.007	0.093	0.516	0.829
20 µM fMLP + 25 µM NVP	**0.002**	**0.049**	0.997	0.997

P values that remained statistically significant after FDR correction are shown in bold. *DMSO* dimethyl sulfoxide, *fMLP* N-formyl-methionyl-leucyl-phenylalanine, *MFI* mean fluorescence intensity, *NVP* nevirapine

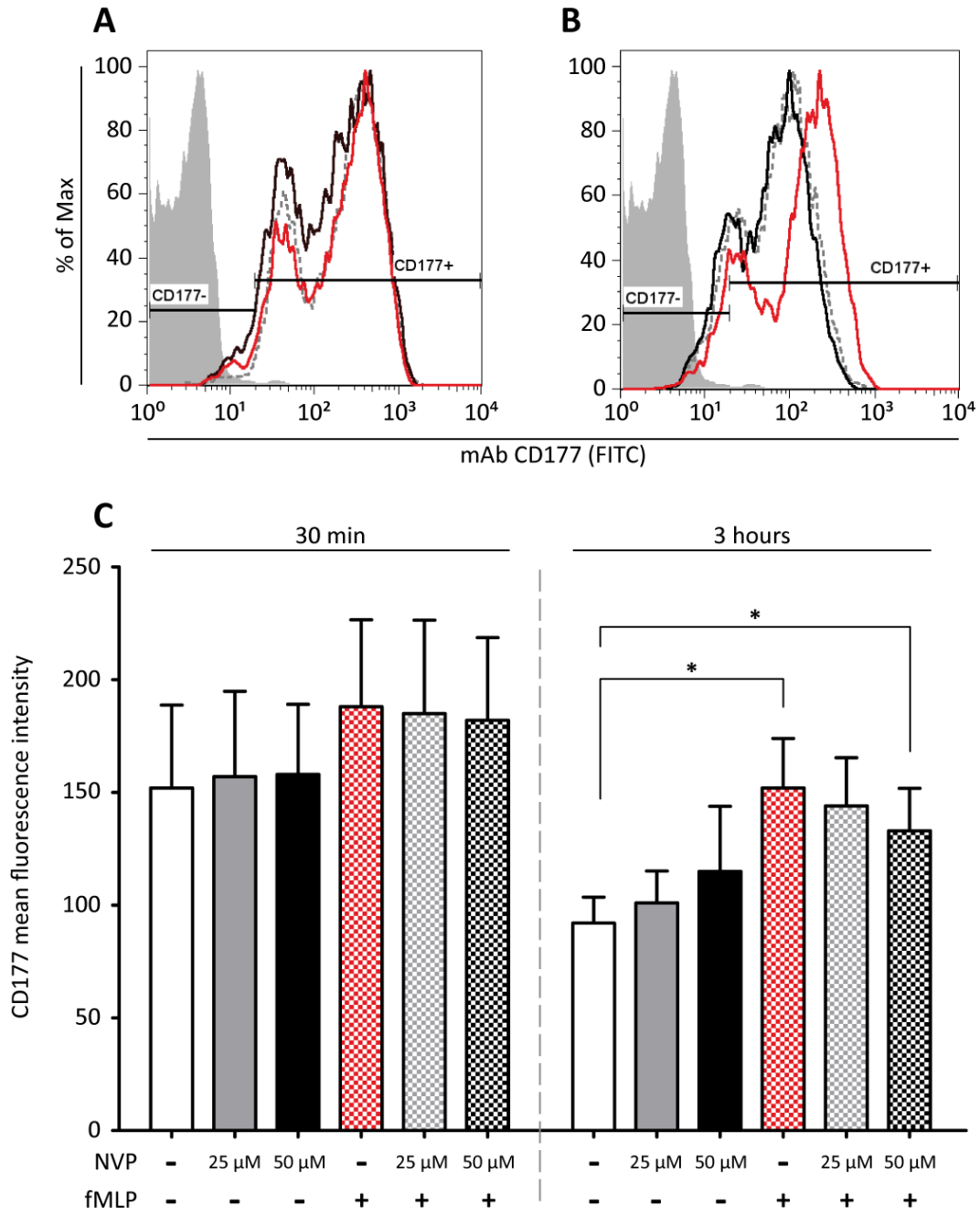


Fig. 2.8: Analysis of CD177 protein expression using flow cytometry in former NVP-hypersensitive patients

Whole blood was treated with 10% DMSO solution, 25 µM and 50 µM NVP as well as 20 µM fMLP as a positive control. Protein expression was analysed with a CD177-specific mAb and surface expression captured as MFI. Typical histograms of one representative patient are shown after treatment for 30 min (**A**) or 3 hours (**B**). Unsorted leukocytes are shown in solid grey. Treatment with 50 µM NVP (black line) results in an overlapping histogram with the DMSO control (dark grey, dashed line). Only the treatment with fMLP (red line) produces a positive peak shift that correlates to an increase in CD177 expression (fig. 2.5 B). The bar graph shows the CD177 MFI of all NVP-treated patients (**C**). A significant increase can only be detected after co-treatment of 50 µM NVP with fMLP or treatment with fMLP alone. Statistical analysis was performed using a paired sample t-test and false discovery rate (FDR) calculated for each test. *fMLP* N-formyl-methionyl-phenylalanine, *mAb* monoclonal antibody, *NVP* nevirapine ;* FDR ≤ 0.05

The percentage of CD177-positve cells remained unchanged after treatment of whole blood with NVP and fMLP at both time points (FDR ≥ 0.828; figure 2.9, table 2.7), indicating that only the amount of antigen expressed in the population changes after *in vitro* treatment.

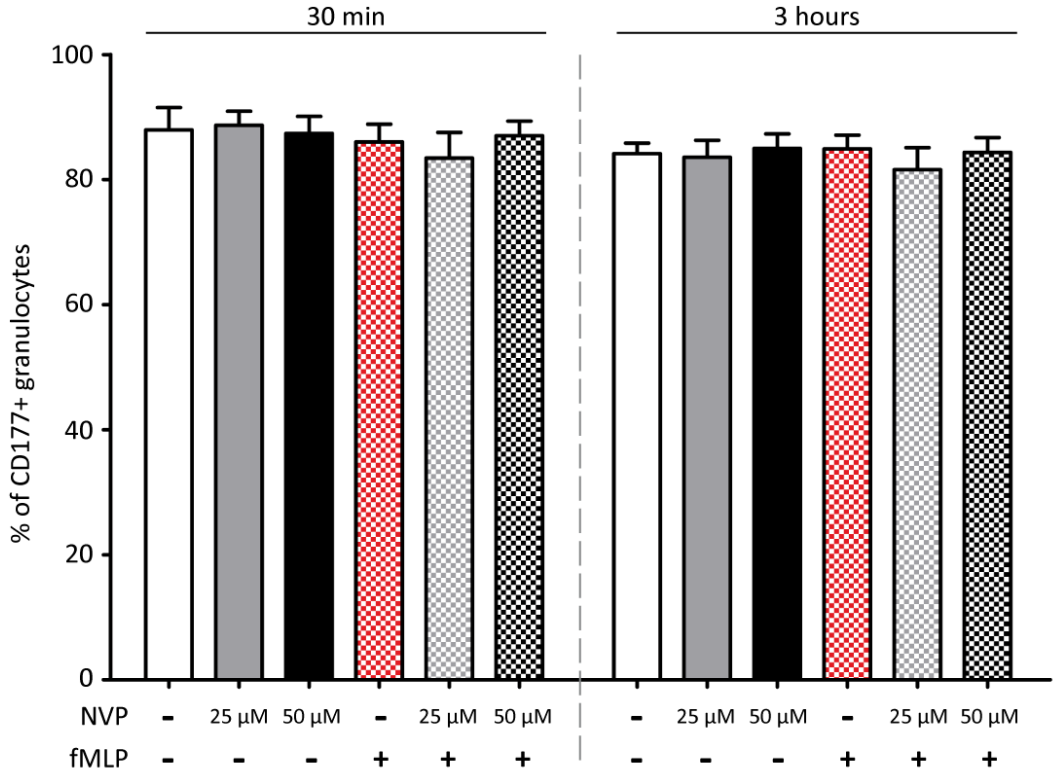


Fig. 2.9: Mean per cent of CD177 positive granulocytes after treatment with NVP and fMLP

No significant difference in the mean population size of CD177 positive granulocytes could be detected after treatment with NVP or fMLP in patient samples collected in Liverpool. *fMLP* N-formyl-methionyl-phenyl-alanine, *NVP* nevirapine,

2.4 Discussion

To validate the microarray data, *CD177* and *PGLYRP1* gene expression profiles of PBMCs from nine NVP-tolerant and 21 hypersensitive black African patients were analysed via qPCR. Expression levels of *CD177* and *PGLYRP1* were both significantly upregulated in tolerant controls when compared to recovered NVP-hypersensitive patients, thus confirming the microarray results that showed a 16-fold increase of *CD177* ($p = 0.003$) and a 2.7 upregulation of *PGLYRP1* ($p =$

0.002) in tolerant patients (manuscript in preparation). In addition, a NVP-dependent induction of these transcripts was shown in NVP-hypersensitive patients during the acute phase of the reaction. Due to the wide inter-individual variability present in the samples, this association could not be detected in the microarray (data not shown). These results were only partially replicated in the *in vitro* data set from Malawi. After treatment with NVP, *CD177* expression was higher in tolerant samples whereas no difference in the expression of *PGLYRP1* could be detected between hypersensitive and tolerant patients. However, none of these results reached statistical significance. The microarray results were further incorporated into a pathway analysis, which revealed *CSF3* to be a functionally relevant member of the CD177 protein interactions (see figure 2.1). Expression levels of *CSF3* could not be detected in 29 of 36 *in vivo* samples, whereas *in vitro* treatment with NVP induced *CSF3* expression in tolerant patients from Malawi. The variation between the *in vivo* and *in vitro* analyses may be based on the small number of *in vitro* samples included in the study, but may also reflect the differences in the experimental setup of both the *in vivo* and *in vitro* samples e.g. incubation time and NVP concentration.

Previous reports examining PRV-*1/CD177* mRNA but not protein expression, have associated increased levels with infection (GOHRING *et al.*, 2004; LILL *et al.*, 2013), inflammation (BUX *et al.*, 1996; LIU *et al.*, 2003; SHAHABI *et al.*, 2013), β-thalassemia (MONTASER *et al.*, 2011) and atypical myeloproliferative neoplasms (SIRHAN *et al.*, 2005). Based on these reports and our observations in patients from Malawi, gene and protein expression of CD177 were investigated *in vitro* in patients and volunteers recruited in the UK. The association of increased *CD177* expression and NVP treatment was confirmed in five retrospectively recruited patients after *in vitro* treatment with NVP. Gene expression analysis showed a NVP-dependent increase in *CD177* expression in patients but not in samples from healthy volunteers. This association only reached statistical significance after treatment with 25 µM and 50 µM NVP for 30 min but not after 3 hours. Taken together, the elevated CD177 mRNA levels observed in NVP-treated patients and *in vitro* treated samples indicate that these changes may either be caused by NVP exposure as both NVP-hypersensitive and tolerant patients showed a statistically significant increase in *CD177* or depend upon the

inflammatory activity reported in HIV patients since expression of CD177 could not be induced in healthy volunteers with NVP (NEUHAUS *et al.*, 2010).

The distinctive expression levels observed between *in vitro* treated patient samples from Malawi and Liverpool are most likely based on the differences in the experimental setup. Whereas samples collected in Liverpool were incubated for 30 min or three hours based on previous studies (see section 2.2.2), the only RNA samples available from Malawi underwent a prolonged exposure to NVP for 24 hours. This may decrease cell viability and thus alter the gene expression profiles detected *in vitro*.

In contrast to the gene expression results, the analysis of CD177 protein expression using flow cytometry did not show a treatment specific effect, which is consistent with previous studies. It has been shown that despite the overexpression of *CD177* mRNA in patients with essential thrombocythemia (STRONCEK, 2007), idiopathic myelofibrosis (TEMERINAC *et al.*, 2000) and polycythemia vera (KLIPPEL *et al.*, 2002; STEIMLE *et al.*, 2007) the amount of CD177 antigen did not differ among patients and healthy volunteers (SLEZAK *et al.*, 2009; TEMERINAC *et al.*, 2000). Our experimental setup was validated by the incorporation of fMLP, a peptide known to enhance neutrophil activation, which showed a significant increase in CD177 antigen expression after three hours. While co-treatment with fMLP and NVP lead to an increased expression of CD177, it can be assumed that this effect is fMLP specific and not dependent on NVP. No difference in the mean population size of CD177-positive granulocytes was observed after treatment with DMSO, NVP or fMLP, which is similar to the results reported by Wolff *et al.* after granulocyte colony-stimulating factor treatment (WOLFF *et al.*, 2003).

In order to confirm if the increase in *CD177* mRNA levels reported in this study acts as a surrogate marker for a pathogenic mechanism for NVP hypersensitivity, further work is needed to elucidate what these factors are. A common pathway may link the regulation of *CD177* mRNA expression with a causative gene involved in the pathogenesis of NVP-induced HSRs. One of the mechanisms leading to an increase in *CD177* gene expression has been linked to the p.V617F polymorphism in the Janus kinase 2 (JAK2). This polymorphism

leads to the constitutive activation of the JAK/signal transducers and activators of transcription pathway [JAK-STAT (JAMES *et al.*, 2005; KRALOVICS *et al.*, 2005)] and has since replaced *CD177* as a diagnostic marker for polycythemia vera (CAMPBELL *et al.*, 2005; JAMES *et al.*, 2005). A comprehensive study of this pathway in combination with other genes differentially expressed in our microarray analysis may result in the identification of central regulators of ADRs associated with NVP.

This study identified significant changes in the transcriptome of NVP-treated, HIV-positive patients and further validated these results in two independent patient cohorts recruited in Malawi and the UK respectively. In addition, the neutrophil specific antigen CD177 was evaluated as a possible blood-borne biomarker for NVP-induced HSRs. *CD177* mRNA was identified as differentially expressed marker in NVP-treated HIV-1 positive patients. Whether this effect is a result of the drug rather then a pre-disposing factor for hypersensitivity will have to be further investigated. Furthermore, pathway analyses based on our results may lead to a better understanding of the molecular processes and biological networks involved in NVP-induced hypersensitivity.

Chapter 3

CD177 gene polymorphisms: analysis of the association with NVP hypersensitivity and CD177 protein expression

Contents

Figures

Tables

3.1 Introduction

CD177 belongs to the Leukocyte antigen 6 supergene family and is located on chromosome 19q13.2 (TEMERINAC *et al.*, 2000). Its open reading frame (ORF) of 1131 bp encodes nine exons and the glycosylphosphatidylinositol-anchored protein is composed of 437 amino acids [see figure 3.1 (BETTINOTTI *et al.*, 2002; CARUCCIO *et al.*, 2006; KISSEL *et al.*, 2002)]. The percentage of CD177-positive neutrophils has been associated with three non-synonymous *CD177* SNPs rs45441892 (c.48G>C), rs10425835 (c.793A>C) and rs61625631 [c.1084G>A (MORITZ *et al.*, 2010; WOLFF *et al.*, 2003)]. Moreover, three additional SNPs have been shown to correlate with the occurrence of both a low and a high CD177-expressing subpopulation instead of only one CD177-positive population: the two non-synonymous SNPs rs45553433 (c.134A>T) and rs78718189 (c.1333G>A,C) as well as the synonymous SNP rs45571738 [c.156G>A (MORITZ *et al.*, 2010)].

In patients with clonal myeloid disorders, the proportion of CD177-positive granulocytes has been shown to be smaller than in healthy volunteers whereas the percentage of CD177-positive neutrophils has been reported to be higher in asthmatic patients (MEYERSON *et al.*, 2013; RAMIREZ-VELAZQUEZ *et al.*, 2013). The expression and percentage of CD177 is also influenced by other physiologic conditions such as pregnancy (CARUCCIO *et al.*, 2003), anti-neutrophil cytoplasmatic antibody (ANCA)-associated vasculitis and systemic lupus erythematosus (HU *et al.*, 2009); it has also been shown to be upregulated after treatment with granulocyte-colony stimulating factor (G-CSF) and fMLP (SACHS *et al.*, 2007) as well as in umbilical cord blood (STRONCEK *et al.*, 1998).

Fig. 3.1 Schematic structure of *CD177* gene and SNPs reported in the literature
Figure shows the organisation of nine exons that comprise the *CD177* gene. Light blue boxes represent the 5' and 3' UTR respectively. Dark blue boxes depict exons. *UTR* untranslated region

Given these associations, the correlation between specific *CD177* genotypes and the occurrence of NVP hypersensitivity were analysed in this chapter. The aims of this study were (i) to replicate the association of *CD177* rs45441892 and rs10425835 with the size of the CD177-positive neutrophil population and (ii) perform an extensive analysis of the genetic variation in *CD177* and its association with NVP-induced adverse events in a black African population from Malawi.

3.2 Methods

3.2.1 Patient populations

Healthy volunteers from Liverpool

Blood samples from 35 healthy volunteers were collected between 2011 and 2012 at the Royal Liverpool and Broadgreen University Hospital. Individuals were of black African (n = 6) and Caucasian (n = 29) ancestry. Written informed consent was obtained from all subjects and data on gender and ethnicity were collected. The study was approved by the North West Research Ethics Committee (UK).

Patients recruited in Malawi

Between 2007 and 2009 a total of 1117 HIV-1 positive, NVP-treated patients were recruited at the Queen Elizabeth Central Hospital in Blantyre, Malawi. The characteristics and design of the study have been previously described in section 2.2.1. A subgroup of 288 patients was randomly selected from this cohort based on DNA and blood sample availability. Of these, 165 patients belonged to the tolerant group whereas 123 patients developed HSRs to NVP.

3.2.2 DNA extraction

Genomic DNA isolation

DNA was isolated from 2 ml EDTA blood using the chemagic DNA Blood kit on the automated chemagic Magnetic Separation Module I (chemagen, Germany) according to the manufacturer's instructions. The magnetic beads used in this kit are based on a polyvinyl alcohol matrix (M-PVA) and exhibit a hydrophilic surface that captures DNA specifically and results in a high yield and purity for each sample. All samples were collected from healthy volunteers and eluted in 500 µl elution buffer.

Spin-column blood DNA extraction of Malawian samples

For DNA extraction up to 250 µl anticoagulated blood was used in the procedure described in the E.Z.N.A. Blood DNA Blood protocol (Omega Bio-Tek, USA). If less than 250 µl blood was available, the volume was brought up to 250 µl using the Elution Buffer provided in the kit. Prior to elution, samples were incubated with 100 µl of preheated Elution Buffer at 70°C for five minutes. The final volume of each sample was decreased to 50 µl using the Vacufuge® vacuum concentrator (Eppendorf, Germany).

3.2.3 Flow cytometry analysis of CD177 baseline expression

A 200 µl aliquot of whole blood, collected in lithium heparin Vacuette® Blood collection tubes (Greiner Bio-One, Germany), was processed as described in chapter 2.2.6 within two hours of venepuncture.

3.2.4 *CD177* genotyping

SNP selection criteria

To investigate the effect of *CD177* polymorphisms on NVP-induced hypersensitivity and CD177 antigen expression, a total of 16 SNPs spanning

17,088 bp across chromosome 19q13.2 were selected. SNPs with a minor allele frequency (MAF) of 5% or greater were identified in the Yoruban population reported by the HapMap consortium [Yoruba in Ibadan, Nigeria (INTERNATIONAL HAPMAP, 2003)]. In addition, SNPs resulting in functional or structural differences in the protein as anticipated by the sequence based characterisation of SIFT (NG and HENIKOFF, 2001) and PolyPhen (JORDAN *et al.*, 2011) were included in this study, as were SNPs that had been previously characterised in the literature or by the National Center for Biotechnology Information [NCBI; (SHERRY *et al.*, 2001)] database and the 1000 Genomes project (GENOMES PROJECT CONSORTIUM *et al.*, 2012).

Genotype quality control

Monomorphic SNPs were excluded from the analysis (n = 2), as were SNPs that deviated from Hardy-Weinberg equilibrium (HWE; p value < 0.01) or had a call rate below 90% (n = 4). If less than 60% of SNPs were genotyped in a sample, these samples were also removed from the analysis.

SNP genotyping analysis using the Sequenom MassARRAY iPLEX platform

PCR and extension primers were designed using the Sequenom MassARRAY Designer software (version 3.1). The software excluded four SNPs due to overlapping sequences of the primers with adjacent SNPs and three more assays were excluded as primers were incompatible for multiplexing due to cross-dimerization. Finally, one multiplex PCR assay was developed, which included 9 SNPs. The sequences of the amplification and extension primers are listed in Appendix 2.1. All oligonucleotides used in the PCR and primer extension were ordered from Metabion GmBH (Germany).

SNP genotyping was performed on the Sequenom MassARRAY iPLEX platform (Sequenom, Germany) according to the manufacturer's protocol. Through the use of Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) mass spectrometry, two levels of specificity are incorporated into the protocol. Following the locus specific amplification of the target sequence by

PCR, the primer extension reaction leads to the incorporation of mass-modified dideoxynucleotide terminators to the oligonucleotide primers. Primers are extended by a single base that is complementary to the target site and the mass of the extended primers is then determined by MALDI-TOF mass spectrometry. The SpetroTYPER software (Sequenom, Germany) automatically transcribes the mass of observed primers into a genotype for each site. For each reaction, 40 ng DNA was added to each well. As part of quality control, genotyping results were compared between duplicated samples and NTCs were included as negative controls.

CD177 genotyping using TaqMan® SNP Genotyping assays

Genotyping of rs45441892 and rs10425835 were performed with TaqMan® SNP Genotyping assays (Applied Biosystems, USA) on DNA samples from 35 healthy volunteers following the manufacturer's instructions. This PCR based assay utilises the 5' nuclease activity of Taq DNA polymerase to distinguish between alleles. Each assay contains a VIC and a FAM labelled probe, which detects one of the SNP-specific alleles by emitting an allele-specific fluorescent signal after degradation of the probe. Fluorescence and allelic discrimination were determined using the SDS software (version 2.2; Applied Biosystems, USA).

Each 5µl reaction consisted of three components: 20 ng genomic DNA, 1 x TaqMan® genotyping master mix and 1x TaqMan® genotyping assay that contains a mix of the respective primers and fluorescent labelled probes. Using 384-well plates, DNA was amplified after an initial enzyme activation step at 95°C for ten minutes. Reactions were run on the Applied Biosystems HT 7900 Fast Real-Time PCR System (Applied Biosystems, USA) as follows: 40 cycles of 15 sec at 95°C and 60 sec at 50°C. Negative controls containing water instead of DNA as well as 10% duplicates were included as part of quality control.

Table 3.1 TaqMan® SNP Genotyping assays

Gene	SNP ID	Allele/Fluorophore		Assay ID
		VIC	FAM	
CD177	rs45441892	C	G	C_27850890_10
CD177	rs10425835	A	C	C_26078441_10

Supplied by Applied Biosystems (USA). *FAM* 6-carboxy-fluorescein

3.2.5 Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics version 20 (IBM Corp., USA). The case-control and haplotype analyses were undertaken using HaploView software [version 4.2; (BARRETT *et al.*, 2005)].

Univariate analysis of association with clinical factors

In healthy volunteers the effect of gender and ethnicity, factors considered to determine CD177 expression, on MFI and per cent of CD177-postive cells was tested using an independent-sample t-test.

Age, gender and CD4+ T cell count were identified as clinical factors that might influence the risk of HSRs in NVP-treated patients. Each factor was assessed individually for association with case/control status. Covariates with a p value < 0.05 were included in the logistic regression model described below. Association with gender, as a binary variable, was evaluated using a Chi-squared test (X^2). For the continuous outcomes (age and CD4+ T cell count) independent-sample t-tests were used to test for any associations. Prior to analysis, CD4+ T cell count was normalised by $\log_{10}$ transformation.

Analysis of the association with MFI and per cent of CD177-positive cells

Sample size determination was performed a priori. Assuming a SNP with 20% MAF, to detect a 20% difference in the proportion of CD177 positive cells between different alleles at 80% power ($\alpha = 0.05$), it was calculated that at least 34 volunteers would be required.

Of the three SNPs (rs45441892, rs10425835 and rs61625631) previously associated with an increase in the percentage of CD177-positive granulocytes, only two assays were commercially available and therefore included in the subsequent analysis. To evaluate the association of rs45441892 and rs10425835 with the MFI and the proportion of CD177-positive cells a one-way analysis of variance (ANOVA) was performed. To correct for multiple testing, the FDR was calculated for each association. In addition, the association between the different expression profiles and the MFI of CD177-positive granulocytes was also tested using an ANOVA.

Logistic regression model

No sample size calculation was performed a priori. Three SNPs (rs45441892, rs4803613 and rs6509088) were tested for association with outcome by fitting two logistic regression models. First, the "baseline model" was fitted which included clinical variables with a p value < 0.05 in the univariate analysis described above. Next, the "genetic model" was fitted which was the same as the first but also included a covariate that encoded the SNP of interest as follows: "0" coded wild-type homozygotes, "1" heterozygotes and mutant-type homozygotes were encoded as "2". This meant that an additive inheritance model was presumed. As only one of our volunteers was homozygous for the mutant allele in rs45441892, it was included in the heterozygous group for analysis purposes and so coded "1". The two models were then compared using the likelihood-ratio test. Thus the association between the SNP and risk of developing NVP-induced HSRs was tested. A Bonferroni correction was used to account for multiple testing and therefore p value < 0.0083 [0.05/6 (three SNPs, two models)] represented a statistically significant result. Patients were excluded from association analysis if genotyping failed or no data on significantly associated clinical outcomes were available.

3.3 Results

3.3.1 Patient demographics

A total of 35 healthy volunteers were recruited in Liverpool of which 19 (54%) were male and 29 (83%) were Caucasians. Six (17%) of the volunteers were of black-African ancestry and included five male and one female individual.

The characteristics of 288 NVP-treated patients recruited in Malawi are listed in table 3.2 below. A total of 123 hypersensitive patients were included in this study. Hypersensitive patients experienced NIR (35%), HSS (14%), SJS (38%) and DILI (13%).

Table 3.2: Clinical profile of 288 NVP-treated patients from Malawi

Covariate		Tolerant (n = 165)	Hypersensitive (n = 123)
Age, median years (IQR)			
		35 (29 - 42)	36 (32 - 43)
Gender, n (%)			
	Male	66 (40)	45 (37)
	Female	99 (60)	78 (63)
Body weight, kg (range)			
		55.2 (48.8 - 60.4)	54.4 (47 - 60)[a]
CD4+ T cell count (at NVP initiation), mean cells/µl (range)			
		168 (71 - 214)	317 (161 - 438)[b]
Hypersensitivity reaction, n (%)			
	NIR	-	44 (35)
	HSS	-	18 (14)
	SJS	-	47 (38)
	DILI	-	16 (13)

[a] body weight and height missing for three patients. [b] CD4 T cell count missing for 5 patients. *IQR* interquartile range. *DILI* drug-induced liver injury, *HSS* hypersensitivity syndrome, *NIR* nevirapine-induced rash, *NVP* nevirapine, *SJS* Stevens-Johnson syndrome

3.3.2 Baseline CD177 expression in healthy volunteers

Several expression patterns were observed (as seen in figure 3.2). In one individual only 10% of CD177-positive cells could be detected (A), whereas six showed a CD177-positive population of 40% or less (B). The majority of volunteers showed an increase in CD177 expression, with a CD177-positive population of 50% (n = 7, C) or more (n = 19, D). In two individuals, over 90% of granulocytes were CD177-positive (E). No statistical difference between the MFI of the different groups could be detected (p = 0.077).

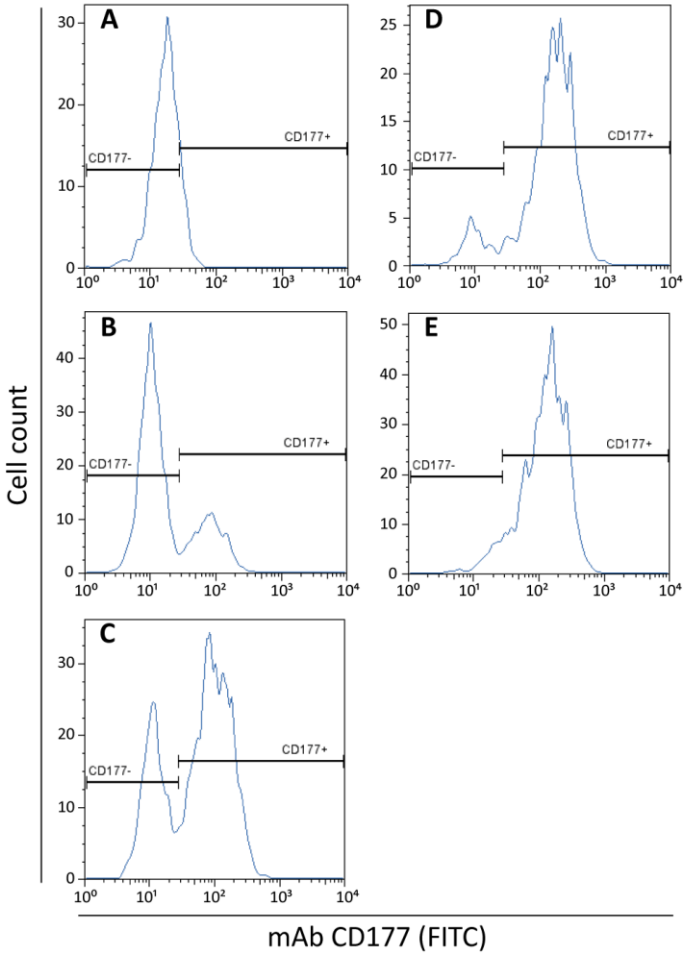


Fig. 3.2: Expression patterns of CD177 on granulocytes from healthy volunteers
The most typical expression patterns featured CD177-negative and CD177-positive subpopulations with varying numbers of cells in each group (≤ 40% **B,** 40-60% **C**, ≥ 60% **D**). Unusual patterns included ≤ 10% (**A**) and > 90% CD177-positive cells (**E**).

3.3.3 Genetic association studies

CD177 SNP associations in healthy volunteers

Neither gender nor ethnicity were significantly associated with the portion or MFI of CD177-positive granulocytes (table 3.3) and were therefore not included in the subsequent analysis.

Table 3.3: Covariates tested in association with the percentage or MFI of CD177-positive granulocytes in healthy volunteers

		P value	
		% of CD177+ granulocytes	MFI of CD177+ granulocytes
Gender	N (%)		
Male	19 (54)	0.116	0.890
Female	16 (46)		
Ethnicity	N (%)		
Black African	6 (17)	0.185	0.511
Caucasian	29 (83)		

MFI mean fluorescence intensity

Figure 3.3 illustrates the distribution of the percentage or MFI of CD177-positive granulocytes across both *CD177* polymorphisms rs10425835 and rs45441982. The MAF and HWE p values of these SNPs are listed in table 3.4 below. The proportion of CD177-positive granulocytes was significantly associated with rs10425835 (p = 0.014) but not rs45441982 (p = 0.076), although a trend towards a higher population could be observed in carriers of the minor C allele (figure 3.3 A and C). None of the two polymorphisms was significantly associated with the MFI of the CD177-positive subpopulation. After correction for multiple testing, the association between the increase in CD177-positive granulocytes and rs10425835 did not remain statistical significant (FDR = 0.055).

Table 3.4: Overview of SNPs and their association in healthy volunteers

SNP	MAF	HWE p value	% of CD177+ granulocytes		MFI	
			P value	FDR	P value	FDR
rs10425835	0.24	0.020	0.014	0.055	0.295	0.351
rs45441892	0.31	0.202	0.076	0.152	0.351	0.351

HWE Hardy Weinberg Equilibrium, *MAF* minor allele frequency, *SNP* single-nucleotide polymorphism

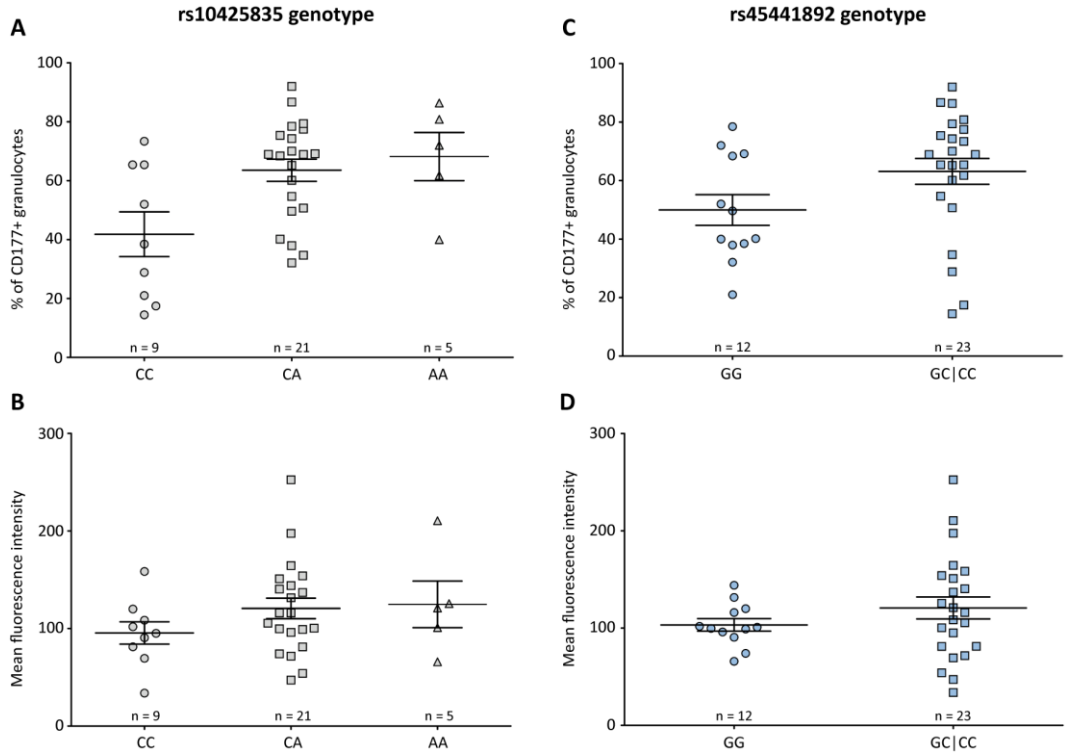


Fig. 3.3: Box and whisker plots showing the distribution of the number and MFI of CD177-positive granulocytes in association with *CD177* rs10425835 and rs45441892 genotypes

The figure shows the distribution of CD177-positive granulocytes and MFI based on the genotype of rs10425835 (**A** and **B**) and rs45441892 (**C** and **D**). Shown is the mean ± standard error. Differences were calculated using a One-Way ANOVA. None of the results were significant after correction for multiple testing although a trend towards a higher proportion of CD177+ granulocytes can be detected for carriers of the minor alleles from SNPs. *ANOVA* analysis of variance, *MFI* mean fluorescence intensity

Association of CD177 SNPs and NVP hypersensitivity

Of the nine SNPs genotyped, two were excluded with a call rate < 90%, two due to a HWE p value < 0.01 and two were monomorphic in our population. Table 3.5 summarizes the SNP data. The remaining three SNPs were listed in the 1000 genomes project (GENOMES PROJECT CONSORTIUM *et al.*, 2012) and gave comparable MAF for black African in our study. Eight patients were excluded from the final analysis; less than 60% of SNPs were genotyped and no CD4+ T cell count was available in three tolerant and five hypersensitive patients respectively.

Table 3.5: Overview of SNPs and allele frequency of nine SNPs genotyped in 285 NVP-patients

dbSNP cluster ID	Gene	Chromosomal position (Chr:bp)	SNP location and function	Alleles (A_1/A_2)	Minor allele	MAF (1000 Genomes database)		MAF (this study)		HWE p value	Reference
						global	African	Tolerant n = 162	All HSRs n = 118		
SNPs included in final analysis											
rs45441892[a]	*CD177*	19:43857873	Exon 1, missense, p.A3P	G/C	C	0.45	0.34	0.33	0.32	0.357	(CARUCCIO *et al.*, 2003; WOLFF *et al.*, 2003)
rs4803613	*CD177*	19:43858317	Intron 2	G/A	A	0.41	0.19	0.16	0.15	0.237	
rs6509088	-	19:43870887	Intergenic, downstream of *CD177*	C/T	C	0.17	0.05	0.06	0.05	0.748	
SNPs excluded as call rate < 90% and or HWE p value < 0.01											
rs10425835[a]	*CD177*	19:43864548	Exon 6, missense, p.L251I	C/A	C	NA		Unknown		0.202	(MORITZ *et al.*, 2010; WOLFF *et al.*, 2003)
rs12462403	-	19:43853799	Intergenic, upstream of *CD177*	G/A	A	0.44	0.35	Unknown		0.0001	
rs7257560	*CD177*	19:43866823	3' UTR	T/C	C	0.42	0.36	Unknown		2.03E-16	
rs73559882	*CD177*	19:43864419	Exon 6, missense, p.F208V	T/G	G	NA		Unknown		0.519	
SNPs excluded as monomorphic											
rs45553433[b]	*CD177*	19:43858044	Exon 2, missense, p.H31L	A/T	T	0.02	0	Unknown		NA	(MORITZ *et al.*, 2010; WOLFF *et al.*, 2003)
rs78718189[b]	*CD177*	19:43866449	Exon 9, missense, p.G431R	G/A	A	0.04	0.01	Unknown		NA	(MORITZ *et al.*, 2010; WOLFF *et al.*, 2003)

[a] SNPs associated with the percentage of CD177-positive neutrophils. [b] SNPs associated with the occurrence of two CD177-positive subpopulations; *Bp* base pair, *Chr* chromosome, *DILI* drug-induced liver injury, *HSS* hypersensitivity syndrome, *HSRs* hypersensitivity reactions, *HWE* Hardy Weinberg Equilibrium, *MAF* minor allele frequency, *NA* not available, *SJS* Stevens-Johnson syndrome, *SNP* single-nucleotide polymorphism, *TEN* toxic epidermal necrolysis, *UTR* untranslated region

Of the clinical factors tested, only CD4+ T cell count was significantly associated with NVP hypersensitivity ($p < 0.001$; see table 3.6) and was thus included in the logistic regression analyses. The mean CD4+ T cell count per patient group is presented in figure 3.4 below.

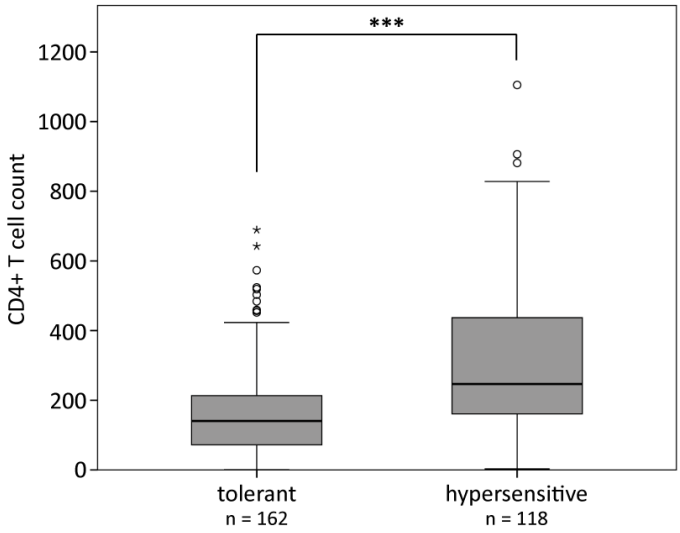


Fig. 3.4: Box and Whisker plot visualising the association between CD4+ T cell count and NVP hypersensitivity in tolerant and hypersensitive patients from Malawi
Boxes represent 25th - 75th percentiles, whiskers show 5th – 95th percentiles, and solid line represents the median and open dots outliers. *** $p < 0.001$

Table 3.6: List of covariates tested for association with NVP hypersensitivity

	Cases		Controls		Difference [95% CI]	p value
	N	Mean (SD)	N	Mean (SD)		
CD4+ T cell count (log_{10})[a]	118	2.37 (0.40)	162	2.03 (0.50)	-0.33 [-0.44, -0.23]	**6.33E-09**
Age[a]	123	38.08 (9.69)	162	36.64 (9.99)	-1.45 [-3.77, 0.88]	0.221
Gender[b]	N	%	N	%		
Male	45	41	64	59	NA	0.615
Female	78	44	98	56		

Significant p values are shown in bold. [a] data analysed using a independent sample t-test, [b] binary data was analysed using a chi-square test. *CI* confidence interval, *NA* not available, *SD* standard deviation

Using a logistic regression model the association between CD177 polymorphisms and risk of NVP-induced HSRs was further examined. Haplotype analysis showed that no LD existed between the SNPs included in this study. None of the SNPs tested showed a significant association with NVP hypersensitivity in our cohort ($p > 0.05$, table 3.7).

Table 3.7: Overview of SNP associations tested using a logistic regression model incorporating CD4+ T cell count as a covariate

rs4803613			
N		Results of Likelihood ratio test	
Hypersensitive	Tolerant	χ^2 (df)	p value
118[a]	160[b]	2.07 (1)	0.150
rs6509088			
N		Results of Likelihood ratio test	
Hypersensitive	Tolerant	χ^2 (df)	p value
118[a]	162	0.11 (1)	0.742
rs45441892			
N		Results of Likelihood ratio test	
Hypersensitive	Tolerant	χ^2 (df)	p value
117[a,b]	161[b]	0.99 (1)	0.318

df degree of freedom, χ^2 Chi-square; [a] samples without CD4+ T cell count were excluded; [b] missing samples failed genotyping

3.4 Discussion

Several studies have mentioned the association between the non-synonymous *CD177* polymorphisms rs45441892, rs10425835 and rs61625631 and an increase in the percentage of CD177-positive granulocytes (MORITZ *et al.*, 2010; WOLFF *et al.*, 2003). In our study of the baseline characteristics in healthy volunteers the mean percentage of CD177-positive granulocytes was significantly upregulated in carrier of the minor A allele (rs10425835) while a trend towards a higher proportion of CD177-positive cells could be observed in carrier of the minor C allele (rs45441892). However, after correction for multiple testing none of these associations retained statistical significance. Although the required sample size was calculated a priori, a larger sample set may have been needed to correct for the high interindividual variability

observed in our cohort of healthy volunteers. The genetic distribution of rs61625631 could not be analysed, as no genotyping assay was commercially available at Applied Biosystems at the time of this study. No effect of gender or ethnicity on the percentage or MFI of CD177-positive granulocytes was detected in our cohort of Caucasian and black African volunteers. This is consistent with the study by Meyerson *et al.* (2013), which suggests that gender did not contribute to the differences in CD177 expression and percentage of CD177-positive granulocytes observed. In addition, a study by Matsuo *et al.* (2000) did not find a significant difference in the percentage of granulocytes between White, Black, Asian and Hispanic patients although gender seemed to be significantly associated with an increase in CD177 expression in this cohort (MATSUO *et al.*, 2000).

Variations in the expression profile of CD177-positive and negative granulocytes have been reported in several studies including our own (MATSUO *et al.*, 2000; MEYERSON *et al.*, 2013; MORITZ *et al.*, 2010; STRONCEK, 2002). To date, no information whether these profiles may affect neutrophil activation and infiltration has been published; the lack of CD177 expression may be based on incomplete mRNA transcription due to incorrect formation of the splicing complex (KISSEL *et al.*, 2002; WOLFF *et al.*, 2003), but further work is needed to elucidate if this is applicable to all patients and how it affects the inflammatory and immunological response.

The occurrence of one or more CD177-positive subpopulations was not investigated as it has been shown that different CD177 antibody clones produce different results (MATSUO *et al.*, 2000; MORITZ *et al.*, 2010) and the 7D8 antibody clone mentioned in the literature was not commercially available at the time of this study. Additionally, various gating strategies and different cut-offs have been reported in the literature which evaluate the difference in CD177 subpopulations, thus preventing a comparative analysis of previously published results.

Furthermore, no associations between the *CD177* polymorphisms (rs45441892, rs4803613 and rs6509088) and NVP hypersensitivity could be observed in our patient group from Malawi. A case-control study failed to show a statistically

significant association of NVP-induced HSRs with rs45441892 (p = 0.166), rs4803613 (p = 0.706) or rs6509088 (p = 0.544). In addition rs45553433 and rs78718189 had to be excluded from the analysis, as they appeared to be monomorphic in our population of Malawian patients. This may be based on the low MAF (< 0.01) reported in Africans and could indicate a lack of power to detect such rare associations in our cohort.

These results support our observation that CD177 is unlikely to be part of the pathomechanisms leading to NVP hypersensitivity, but the increase in *CD177* expression may rather function as a surrogate marker for a mechanistic element, which has yet to be identified (see chapter 2). Whether a correlation between baseline or post-treatment mRNA levels and *CD177* polymorphisms existed in our patient cohort could not be determined due to small overlap of samples included in both studies (n = 7, 14 and 8 for tolerant, acute and recovered samples respectively).

Within our patient cohort, CD4+ T cell count was the only statistically significant risk factor for NVP-induced ADRs. In the logistic regression model, mean CD4+ T cell count was higher in NVP-hypersensitive patients compared to tolerant controls (317 cells/µl and 168 cells/µl respectively). These results are consistent with other studies that have described CD4+ T cell count as a significant risk factor of NVP-induced hypersensitivity (BERSOFF-MATCHA *et al.*, 2001; BOEHRINGER-INGELHEIM, 2012; LYONS *et al.*, 2006; STERN *et al.*, 2003).

In conclusion, no statistically significant association between the proportion of CD177-positive granulocytes or the MFI and the rs10425835 or rs45441892 polymorphisms could be detected in a group of Caucasian and black African healthy volunteers. In addition, none of the SNPs showed a significant association with NVP hypersensitivity in our cohort of black African NVP-treated patients from Malawi. Thus *CD177* genotype is unlikely to contribute to the risk of NVP-induced hypersensitivity directly, which is consistent with the observations made in chapter 2.

Chapter 4

Expression analysis of miR-148a in NVP-treated tolerant and hypersensitive patients

Contents

Figures

Tables

4.1 Introduction

To date, over 30.000 mature miRNAs have been identified across 206 species with more than 2500 of these being expressed in humans [http://www.mirbase.org, release 20 (GRIFFITHS-JONES, 2006)]. MiRNAs have been suggested to regulate the expression of approximately 30% of protein-coding genes, and are often expressed in a tissue specific or developmental-stage specific pattern. Each miRNA targets an average of 200 mRNAs and each mRNA can be regulated by various miRNAs (reviewed in KROL *et al.*, 2010). In the nucleus, miRNAs are transcribed from either independent genes or as introns of protein-coding transcripts (miRtrons) by the RNA polymerases Pol II or Pol III (as shown in figure 4.1). Cleavage of miRtrons by the spliceosome or processing of primary precursor miRNAs (pri-miRNAs) by the Drosha RNase protein complex leads to the formation of a 70-nucleotide long precursor miRNA (pre-miRNA). Subsequently, the pre-miRNA is transported to the cytosol by Exportin 5 where processing by Dicer, another RNase, results in a miRNA duplex molecule. One strand of the miRNA duplex is incorporated into the RNA induced silencing complex (RISC) while the other strand is degraded. RISC is then directed by the miRNA to its 3' UTR binding site. Through imperfect base pairing between the miRNA and the complementary mRNA target, translational repression is initiated inhibiting protein synthesis. If the miRNA sequence is nearly 100% complementary to its binding site, mRNAs are degraded directly through enzymatic cleavage (OCHS *et al.*, 2011).

In HIV-positive patients, 'high expression' HLA-C alleles have been shown to provide increased protection during the progress of HIV infection (APPS *et al.*, 2013; THOMAS *et al.*, 2009). Differences in HLA-C protein expression can be partially explained by binding of miR-148a to the 3' UTR of "inhibited" *HLA-C* alleles (*HLA-C*01, C*03, C*04, C*07, C*14 and C*17*), which do not contain the 263del mutation (KULKARNI *et al.*, 2011; O'HUIGIN *et al.*, 2011).

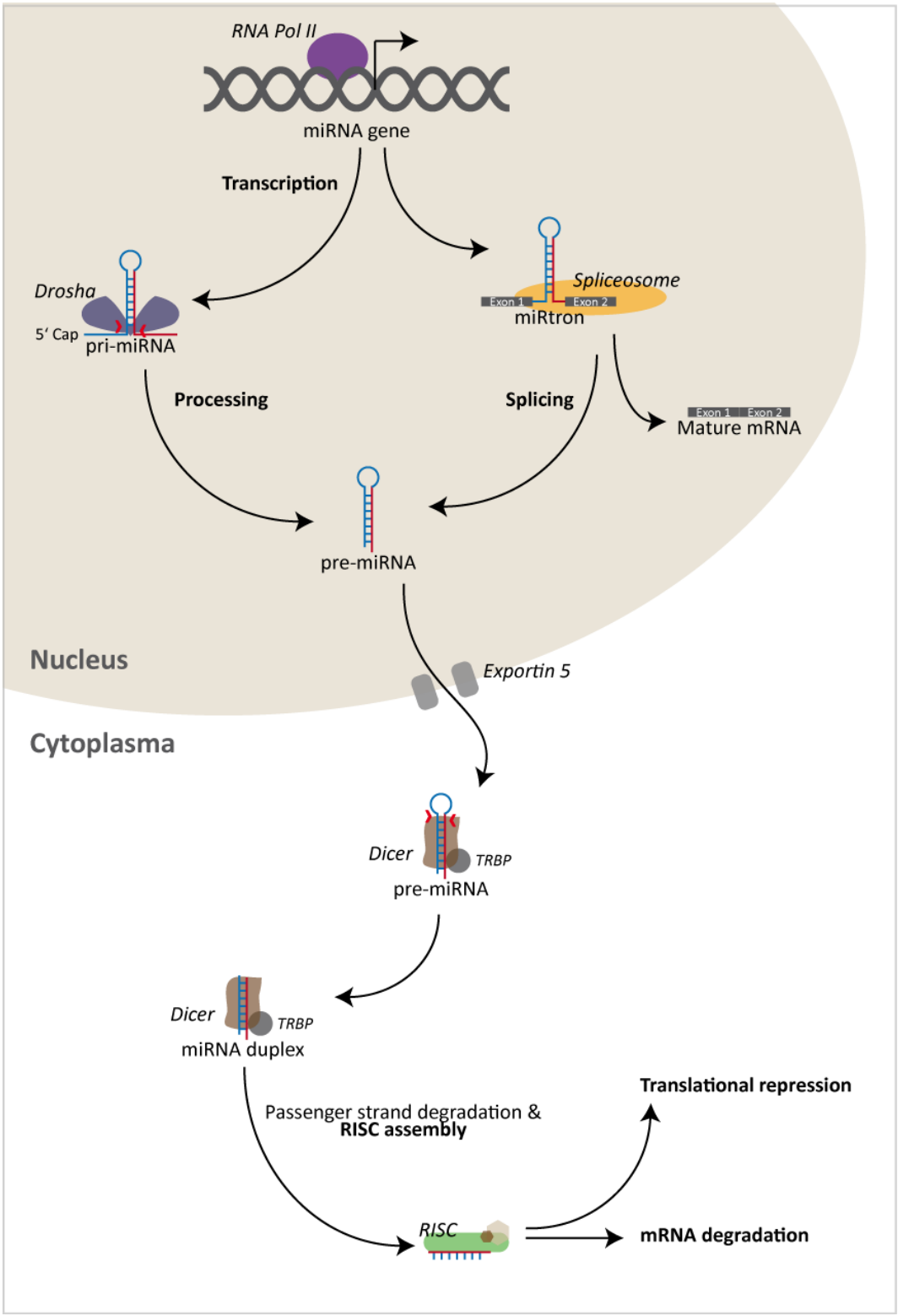


Fig. 4.1: miRNA biogenesis and mechanisms of action

Transcribed by Pol II or Pol III from independent genes or introns of protein-coding genes, miRNAs are processed into 70 nucleotide pre-miRNAs by the spliceosome for miRtrons or from pri-miRNAs by the Drosha protein complex. Exportin 5 transports pre-miRNAs from the nucleus into the cytosol where Dicer together with TRBP processes the miRNAs into a miRNA duplex. Next, the guide strand of the miRNA duplex is incorporated into the RNA-induced silencing complex (RISC) that mediates mRNA degradation or translational repression. The passenger strand is degraded (KROL *et al.*, 2010). *Pol* RNA polymerase, *pre-miRNA* precursor miRNA, *pri-miRNA* primary miRNA, *TRBP* transactivation responsive RNA-binding protein

In our studies, *HLA-C*04:01* was significantly associated with an increased risk of NVP hypersensitivity [OR 2.64 (95% CI: 1.13, 6.18)] and specifically NVP-induced SJS/TEN in patients from Malawi [OR 17.52 (95% CI: 3.31, 92.8); (CARR *et al.*, 2013)]. These results are further supported by a GWAS performed in a combined cohort of NVP-treated patients from Malawi, Uganda and Mozambique that identified a significant association of a SNP located within the *HLA-C* locus and SJS/TEN (OR 5.17, $p = 2.6 \times 10^{-10}$; manuscript in preparation). Atypical expression patterns of miRNAs have been reported in different diseases including asthma (miR-146a, JIANG, 2011), heart disease (IKEDA *et al.*, 2007), skin inflammation (SONKOLY *et al.*, 2008), inflammatory bowel disease (ZWIERS *et al.*, 2012) and rheumatoid arthritis (PAULEY *et al.*, 2008). Based on these observations we hypothesised that miR-148a expression may be altered in NVP-treated patients and thus not only the presence of *HLA-C*04* but also post-transcriptional regulation of *HLA-C* and consequently HLA-C protein expression may be associated with NVP-induced HSRs. The specific aims of this chapter were to (i) establish and validate an analytical method for the detection of miRNAs in serum of HIV positive patients; to examine (ii) any differences in miR-148a expression in serum samples from hypersensitive and tolerant patients at various time points of NVP treatment, and to (iii) investigate protein expression of HLA-C in healthy volunteer samples after treatment with NVP *in vitro*.

4.2 Methods

4.2.1 Patient populations

Malawi cohort samples

Of the 1117 NVP-treated patients recruited in Blantyre (Malawi), serum samples of 30 NVP-hypersensitive patients, who developed cADRs and multi-system reactions, were randomly chosen for this analysis. The characteristics of the main cohort are described in chapter 2.2.1. Additionally, 42 NVP-tolerant patients were chosen as controls and were if possible matched for age and

gender to reduce confounding factors; several cases were matched to more than one tolerant control.

A total of 43 baseline samples (referring to samples taken before ART initiation) were available for both hypersensitive (n = 16) and tolerant patients (n = 27). In addition, 26 acute hypersensitive samples were available; 27 week 6 tolerant samples were chosen as controls, as none of the hypersensitive patients included in this analysis developed HSRs later than six weeks after treatment initiation. These samples were referred to as post-treatment samples. Thus, a total of 96 patient samples were used in this study.

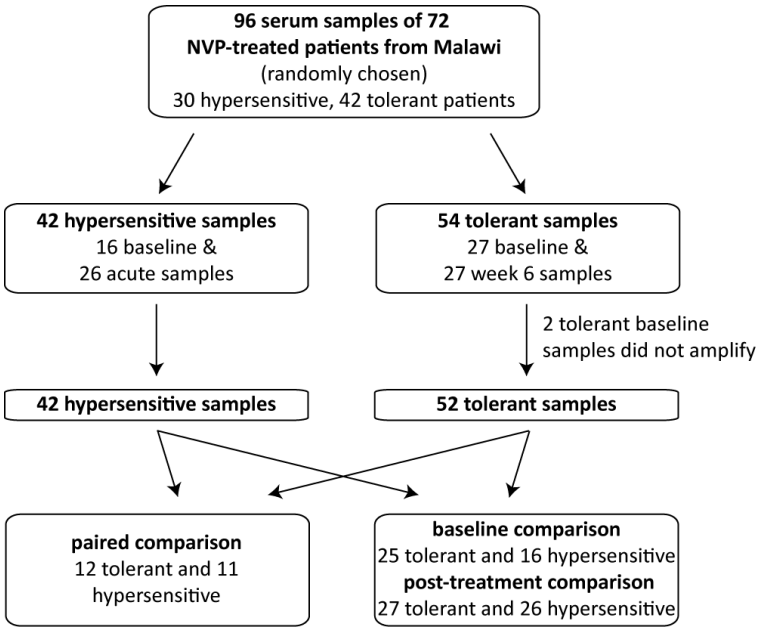


Fig. 4.2: Overview of serum samples from NVP-treated patients used for miR-148a expression analysis

Healthy volunteers recruited at SAIC-Frederick, Inc.

Six healthy, black volunteers were recruited at the Frederick National Laboratory for Cancer Research, operated by the SAIC-Frederick, Inc. (now Leidos Biomedical Research, Inc.) in Maryland, USA. Three of the healthy volunteers were homozygous for the "inhibited" *HLA-C*04:01*, while samples from homozygous *HLA-C*06:02* ("escape" allele) volunteers were used as controls.

4.2.2 RNA extraction & quality control

Whole blood collected in Vacutainer® SST serum tubes (BD™ Biosciences, USA) was inverted several times and centrifuged at 1400 rpm for 15 min. The serum layer was collected, aliquoted and stored at -80°C. Part of the samples (40 out of 96) had been heat-inactivated at 58°C for 40 min for previous experiments. As serum samples had to be extracted in batches, hypersensitive samples and matched controls were varied in order to avoid bias. This was maintained throughout the extraction and qPCR procedure described below.

Serum samples were thawed on ice. Total RNA was extracted from four different serum samples ("preliminary experiment") using three different kits [mirVANA™ PARIS™ kit (Lifetechnologies, USA), Zymo Direct-zol™ RNA MiniPrep (Zymo Research, USA) and QIAGEN miRNA Serum & Plasma kit (QIAGEN, Germany)] to establish performance on small RNA molecules (table 4.1). All kits were used according to the manufacturer's instructions with the following modifications:

- prior to elution, the filter of the Ambion mirVANA™ PARIS™ kit (Lifetechnologies, USA) was spun for two minutes instead of one;
- using the Zymo Direct-zol™ RNA MiniPrep (Zymo Research, USA), total RNA was eluted twice in 30 µl of RNase-free water.

RNA integrity was analysed using the Agilent 2100 Bioanalyzer and the Agilent Small RNA kit (Agilent Technologies, USA) as well as the NanoDrop 1000 spectrophotometer (Thermo-Fischer Scientific, USA). Each sample was extracted twice and RNA was eluted in 14 µl RNase-free water. All RNA samples were stored at -80°C.

Previous studies have shown that the quantity and quality of miRNAs cannot be assessed accurately by spectro-photometry (KROH *et al.*, 2010) and even the evaluation of serum miRNAs can be difficult as none of the cellular RNAs can be used as reference (reviewed in: PRITCHARD *et al.*, 2012). Thus, a fixed volume of eluted RNA was used for normalisation of samples instead of a fixed amount of RNA and a synthetic, *C. elegans* miRNA (cel-miR-39; supplied by QIAGEN, Germany) was spiked-in after the initial denaturation step with QIAzol.

4.2.3 TaqMan® miRNA real-time polymerase chain reaction

Reverse transcription of miRNAs

RNA samples were thawed on ice. Three microliters of total RNA was reverse transcribed to cDNA using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, USA) together with miRNA specific RT primers (stem-loop primers; see table 4.1) according to the manufacturer's instructions. A 15 µl reaction containing 1.5 µl Reverse Transcription Buffer, 1 µl Multiscribe™ Reverse Transcriptase, 0.15 µl dNTPs, 0.19 µl RNase Inhibitor, 6.16 µl RNase-free water, 3 µl miRNA-specific RT primer and 3 µl RNA (equivalent to 43 µl serum) was added to a 96-well plate. Reverse transcription was performed on the Veriti® Thermal Cycler (Applied Biosystems, USA) under the following conditions: 30 min at 16°C, 30 min at 42°C and 5 min at 85°C. The cDNA was stored at -20°C until further use.

In addition to the internal reference miRNA cel-miR-39, four previously established miRNAs were included in a "preliminary experiment" to evaluate their efficiency as invariant, endogenous controls: two small, noncoding RNAs RNU6B (SHARKEY *et al.*, 2012) and RNU48 (STARKEY LEWIS *et al.*, 2011) as well as miR-16 (KROH *et al.*, 2010) and miR-21 [table 4.1; (SHAFFER *et al.*, 2012)]. Endogenous controls with C_T values > 36 cycles were excluded from further analysis.

Table 4.1: TaqMan® MicroRNA assays

Assay Name	Assay ID	miRBase ID (version 20)/ NCBI Accession #	Mature miRNA sequence
hsa-miR-148a	000470	hsa-miR-148a-3p	UCAGUGCACUACAGAACUUUGU
hsa-miR-16	000391	hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG
hsa-miR-21	000397	hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA
RNU6B	001093	NR_002752	CGCAAGGATGACACGCAAATTCGTGAAGCGT TCCATATTTTT
RNU48	001006	NR_002745	GATGACCCCAGGTAACTCTGAGTGTGTCGCT GATGCCATCACCGCAGCGCTCTGACC
cel-miR-39	000200	cel-miR-39-3p	UCACCGGGUGUAAAUCAGCUUG

Supplied by Applied Biosystems (USA). MiRNAs that were included in the final experimental setup are shown in bold.

qPCR amplification

TaqMan® MicroRNA assay utilises the same technologies as described for TaqMan® Gene Expression assays in section 2.2.4. The 20 µl reaction containing 10 µl TaqMan® Universal PCR Master Mix II, 1 µl Small RNA Assay, 7.7 µl RNase-free water and 1.3 µl cDNA was added to a 96-well plate. All samples were run in duplicate and NTCs for each miRNA were added as part of quality control. The plate was run on the Applied Biosystems HT 7900 Fast Real-Time PCR system (Applied Biosystems, USA) as follows: 10 min at 95°C followed by 40 cycles of a denaturing step at 95°C for 15 seconds and an annealing/extension step at 60°C for 1 min. Prior to data extraction, the threshold was set to 0.03 for all miRNAs.

4.2.4 C_T normalisation procedure

Data was analysed using SDS software (version 2.2; Applied Biosystems). Two protocols have been proposed for normalisation of experimental qPCR data from serum and plasma samples using spiked-in synthetic, non-human miRNAs (KROH *et al.*, 2010; MCDONALD *et al.*, 2011; SHAFFER *et al.*, 2012). These will be explained in further detail below. After the primary amplification test, results for miR-16, miR-21 and miR-148a were calculated using both techniques before the most suitable miRNA was chosen as the endogenous control (section 4.3). Results of both methods were compared.

Median normalisation procedure

The method proposed by Kroh *et al.* (2010) and McDonald *et al.* [(2011); summarised below] required the calculation of a mean C_T for cel-miR-39 of each individual sample. Next the median of all cel-miR-39 C_T values was calculated. A normalisation factor was calculated by subtracting the median C_T of each individual mean cel-miR-39 value. This normalisation factor was then subtracted from the mean C_T value of the target gene of each sample.

$$\text{target miRNA}_{normalised} =$$

$$\text{target}_{raw} - [\text{cel-miR-39}_{mean\ CT\ of\ given\ sample} - \text{cel-miR-39}_{median\ CT\ all\ samples}]$$

Calibration using a synthetic C. elegans control

The mean cel-miR-39 C_T for each of the following groups was calculated: tolerant baseline, tolerant week 6, hypersensitive baseline and hypersensitive acute. For calibration, the difference between the mean C_T value of the hypersensitive baseline group and each of the remaining groups was calculated. This calibration factor was then added to every sample in the respective group (table 4.2, SHAFFER *et al.*, 2012). The level of miRNA expression was calculated using the $2^{-\Delta C_T}$ method as described in section 2.2.5.

Table 4.2: Mean C_T values and calibration factors established using the method by Shaffer *et al.* (2012)

Group	Mean C_T	Calibration factor Hyp baseline C_T – group C_T
Tolerant baseline	20.74	0.435
Tolerant week 6	20.06	1.112
Hypersensitive baseline	21.17	-
Hypersensitive acute	20.19	0.978

4.2.5 Leukocyte preparation and *in vitro* culture

Whole blood collected in Vacutainer® EDTA tubes (BD™ Biosciences, USA) was diluted 1:1 with phosphate buffered saline (PBS; Lonza Walkersville, USA) before the solutions were mixed by inversion. Fifteen millilitres of Lymphocyte separation medium (Lonza Walkersville, USA) was then overlayed with the blood/PBS solution and centrifuged for 30 min at 1650 rpm. The interface containing the lymphocytes was then transferred to a new tube and washed by adding 25 ml of PBS. The solution was then spun for ten minutes at 1000 rpm and washed twice more before the number of cells was quantified using a Hemocytometer. Fifteen million cells were added to a six well plate and

incubated with either 25 µM NVP or a 10% DMSO solution for 24 hours under standard cell culture conditions (37°C, 5% CO_2).

4.2.6 Flow cytometry

HLA-C surface expression was investigated by labelling lymphocytes first with an unlabelled HLA-C specific antibody [mouse anti-human (clone DT9, 3.73 dilution); kindly provided by Dr. Richard Apps] that was further bound by a PE-labelled goat anti-mouse antibody (clone UPC-10; Sigma, USA). A total of $x10^5$ cells were washed by addition of 3 ml FACS buffer [PBS + 1% fetal bovine serum (Lonza Walkersville, USA)] and centrifuged at 450 g for five minutes. After centrifugation, the primary antibody or isotope control were added to each tube and incubated for 30 min at 4°C. Cells were washed as described above before a secondary anti-mouse, PE-labelled antibody was added. After incubation for 30 min at 4°C, unbound antibody was removed by washing with FACS buffer. After the last centrifugation step, cells were resuspended in 500 µl FACS buffer and the fluorescent signal was acquired on the FACS Canto II system using the FACSDiva software (version 6.1.2; BD™ Biosciences, USA). Data were analysed using the FlowJo version 8.7 software (TREE STAR INC., 2013). A representative example of the gating strategy and histogram is shown in figure 4.3 below.

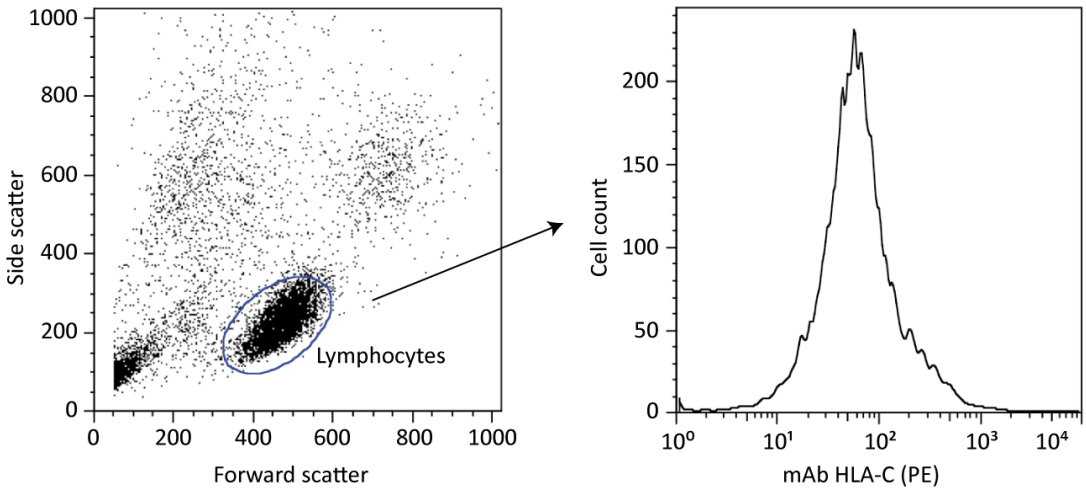


Fig. 4.3: Gating strategy for HLA-C expression analysis
Lymphocytes are gated on a forward scatter/side scatter dot plot before events are counted on a HLA-C histogram. Unspecific binding is calibrated using PE-isotope control antibodies; a higher fluorescence suggests specific antibody binding of the target antibody.

4.2.7 Statistical analysis

All statistical analyses were performed using the IBM SPSS Statistics version 20 (IBM Crop., USA). The correlation analysis was performed using GraphPad Prism (version 5.01 or Windows, GraphPad Software, USA, www.graphpad.com). A p value < 0.05 was regarded as statistically significant if not otherwise stated. If the amplification of miRNAs failed, samples were excluded from the analysis.

Univariate analysis

The following factors were identified as clinical factors that might influence miR-148a expression levels in NVP-treated patients: CD4+ T cell count, carriage of *HLA-C*04:01* or any other *HLA-C* allele known to be regulated by miR-148a or heat-inactivation of samples. To evaluate the effect of each factor on miR-148a expression, univariate linear regression analysis was performed.

Analysis of miR-148a expression

Since data were not normally distributed and none of the covariates were significantly associated in the linear regression analysis, expression analysis was performed using non-parametric tests.

The difference between baseline and post-treatment samples was analysed in paired samples from either tolerant (baseline versus week 6 controls) or hypersensitive patients (baseline versus acute samples) using a Wilcoxon test. Further, a Kruskal-Wallis test was used to test the difference between tolerant and the different hypersensitivity phenotypes at baseline or post-treatment. Finally, the difference in expression between tolerant and hypersensitive patients (calculated as the difference between paired post-treatment and baseline samples) was evaluated using the Mann-Whitney U test. After correction for multiple testing, a Bonferroni threshold of $p = 0.05/5 = 0.01$ (five tests) was regarded as statistically significant.

4.3 Results

The amplification of two tolerant baseline samples failed, which were therefore excluded from all further analysis. A total of 94 patient samples were used in this study. Fifty-seven of the 94 samples included in the analysis were HLA genotyped; for the remaining samples not enough high-quality DNA was available for genotyping.

4.3.1 Population characteristics

Characteristics of the 72 NVP-treated patients recruited in Malawi are outlined in table 4.3 below. Fifty-five per cent tolerant and 63% of hypersensitive samples were female. Mean CD4+ T cell count and body weight were 196 cells/µl and 53.9 kg in tolerant controls, while hypersensitive patients featured a mean CD4+ T cell count of 218 cells/µl and body weight of 52.1 kg. The median age was 36 years for tolerant and 37 for hypersensitive patients.

Sixteen (53%) of the 30 hypersensitive patients developed NIR, while six (20%) experienced HSS and eight (27%) experienced SJS.

Table 4.3: Demographic data of NVP-treated patients

Covariate	Tolerant (n = 42)	Hypersensitive (n = 30)
Age, median years (IQR)		
	36 (32 – 41)	37 (32 – 42)
Gender, n (%)		
Male	19 (45)	11 (37)
Female	23 (55)	29 (63)
Body weight, kg (range)		
	53.9 (47.0 – 57.8)	52.1 (45.1 – 58.4)[a]
CD4+ T cell count (at NVP initiation), mean cells/µl (range)		
	196 (101 – 260)	218 (120 - 250)
Hypersensitivity reaction, n (%)		
NIR	-	16 (53)
HSS	-	6 (20)
SJS	-	8 (27)

[a] body weight is missing for one hypersensitive patient. *IQR* interquartile range, *HSS* hypersensitivity syndrome, *NIR* nevirapine-induced rash, *NVP* nevirapine, *SJS* Stevens-Johnson syndrome

4.3.2 Optimisation of miRNA analysis

MiRNA extraction and choice of endogenous control

Due to the inadequate accuracy of quality and quantity measurements on RNA extracted from serum samples using spectro-photometry, we performed the final extraction of all serum samples using the QIAGEN miRNA Serum & Plasma kit (QIAGEN, Germany) based on our results from the Bioanalyzer Agilent Small RNA kit (results are summarised in table 4.4). RNA was extracted from 200 µl serum.

Table 4.4: Overview of results from miRNA extraction

Kit	Amount of serum used [µl]	Bioanalyzer miRNA concentration [pg/µl]	NanoDrop Concentration [ng/µl]
Zymo Direct-zol™ RNA MiniPrep	100	10.28	2.91
Ambion mirVANA™ PARIS™ kit	400	62.95	17.01
QIAGEN miRNA Serum & Plasma kit	200	**233.40**	5.45

Preliminary data demonstrated that both RNU6B and RNU48 featured C_T values > 36 cycles and as such were excluded from further analysis (data not shown). The software package NormFinder (ANDERSEN *et al.*, 2004) was used to determine the ideal normalization gene, choosing between miR-16 and miR-21. MiR-16 showed the lowest inter-group variability (stability value = 0.018) and was chosen as endogenous control for all further experiments.

Comparison of CT normalisation procedures

As shown in figure 4.3 the normalised C_T values for 94 samples, calculated using the median normalisation procedure (MCDONALD *et al.*, 2011) or calibrated to the spiked-in cel-miR-39 (SHAFFER *et al.*, 2012), showed a strong positive correlation for miR-16 ($r = 0.714$, $p < 0.0001$; figure 4.4 A) and miR-148a ($r = 0.686$, $p < 0.0001$; figure 4.4 B). Based on these results we focused all further

analyses on the results that were calculated using the method proposed by McDonald *et al.* (2011).

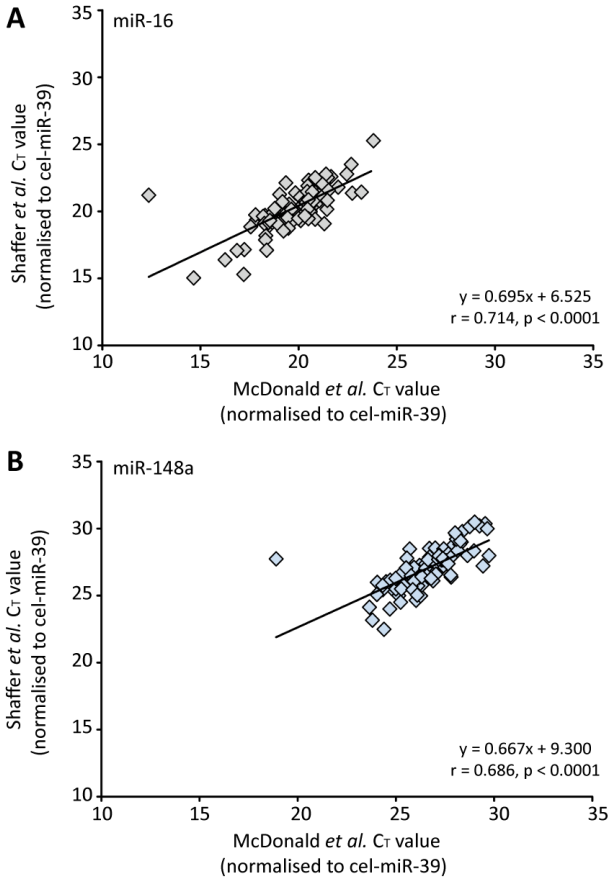


Fig. 4.4: Correlation between C_T values calculated using two different techniques

C_T values were normalised to cel-miR-39 using the Shaffer *et al.* or McDonald *et al.* normalisation procedure. Pearson rho correlation and p value are given for miR-16 (**A**) and miR-148a (**B**) as well as the slope-intercept equation. C_T values of both miRNAs show a strong positive correlation. n = 94

4.3.3 Differential miR-148a expression in NVP-treated patients

Results of the linear regression analysis to explore the effect of clinical covariates on miR-148a expression levels are listed in table 4.5. Neither CD4+ T cell count (p = 0.549), carriage of *HLA-C*04:01* (p = 0.968) or other *HLA-C* alleles regulated by miR-148a (p = 0.198), nor heat-inactivation of samples (p = 0.463) were significantly associated with the miR-148a expression and were consequently not adjusted for in the analysis of association between NVP-induced hypersensitivity and miR-148a expression.

Table 4.5: Covariates were tested for association with miR-148a expression using a linear regression model

Covariate	Total n = 94 samples	P value
CD4+ T cell count (at NVP initiation), mean cells/ µl (range)		
	212 (120 – 258)	0.549
HLA-C*04:01 **carrier, n (%)**		
	23 (24)[a]	0.968
Carrier of *HLA-C* alleles controlled by miR-148a†, n (%)		
	48 (49)[a]	0.198
Heat-inactivation		
	43 (44)	0.463

None of the covariates was significantly associated with miR-148a expression. [a] HLA genotyping available for 57 of 94 samples. † "inhibited" alleles include: *HLA-C*01, C*03, C*04, C*07, C*14* and *C*17.*

Using a Wilcoxon test, the difference between baseline and post-treatment miR-148a expression was analysed in paired samples from tolerant (n = 11) or hypersensitive patients (n = 12; figure 4.5). The qPCR analysis showed that miR-148a was significantly upregulated during the acute phase of reaction in NVP-hypersensitive patients (p = 0.008), but not after six weeks of NVP treatment in tolerant controls (p = 0.092) although a small increase can be observed in post-treatment samples. Results remained statistically significant after correction for multiple testing (significant threshold = 0.05/5 = 0.01).

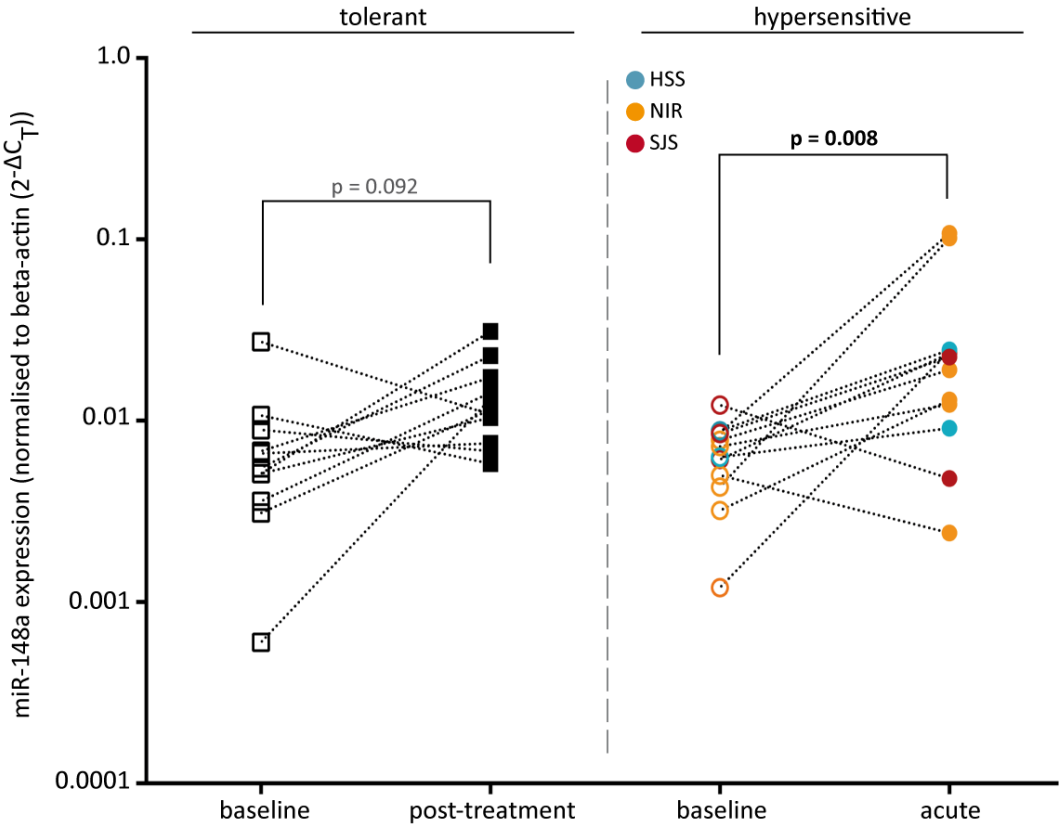


Fig. 4.5: miR-148a expression analysis in paired tolerant or hypersensitive samples
Expression between paired baseline and post-treatment samples were compared in tolerant (left panel; n = 11) and hypersensitive patients (right panel, n = 12). Post-treatment patient samples were taken six weeks after treatment initiation in tolerant and during the acute phase of the adverse event in hypersensitive patients. A significant difference was detected between baseline and acute hypersensitive samples. Data was analysed using a Wilcoxon test and results remained significant after correction for multiple testing. *HSS* hypersensitivity syndrome, *NIR* nevirapine-induced rash, *NVP* nevirapine, *SJS* Stevens-Johnson syndrome

Further, the difference in miR-148a expression levels from tolerant and hypersensitive patient samples was analysed either at baseline or post-treatment using a Kruskal-Wallis test. No statistically significant difference in miR-148a expression levels was detected between tolerant and hypersensitive patients at baseline (p = 0.577) nor post-treatment (p = 0.349; figure 4.6). Additionally, the difference in miR-148a expression, calculated as the difference between paired post-treatment and baseline samples, was analysed between tolerant controls and NVP-hypersensitive patients using the Mann-Whitney U test. There was no statistically significant difference between both groups (p = 0.242; figure 4.7).

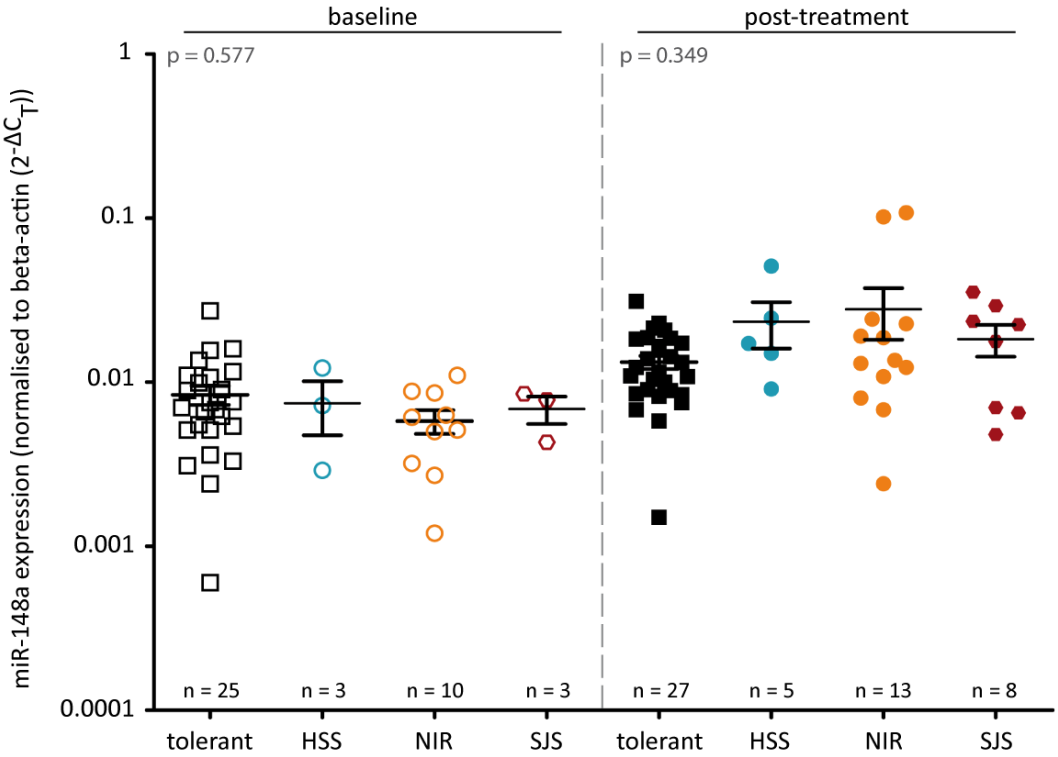


Fig. 4.6: Expression analysis of miR-148a at baseline or post-treatment

miR-148a expression was compared between hypersensitive and tolerant patient samples at either baseline (left panel) or post-treatment with NVP (right panel). No difference was detected between the different hypersensitive samples and tolerant controls at both time points. Analysis was performed using a Kruskal-Wallis test. *HSS* hypersensitivity syndrome, *NIR* nevirapine-induced rash, *NVP* nevirapine, *SJS* Stevens-Johnson syndrome

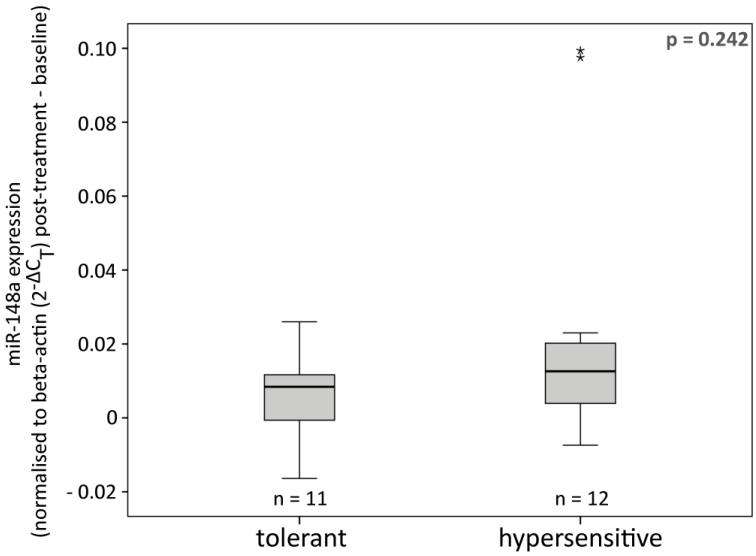


Fig. 4.7: Comparison of the difference in miR-148a expression between tolerant and hypersensitive patients

Difference in expression was calculated by subtracting the baseline ratio from the post-treatment ratio (calculated using $2^{-\Delta C_T}$). No difference between tolerant and hypersensitive patients could be detected. Data was analysed using a Mann-Whitney U test.

4.3.4 HLA-C in vitro expression analysis

Using flow cytometry, differences in the HLA-C expression were analysed in lymphocytes from *HLA-C*04:01* and *HLA-C*06:02* homozygous healthy volunteers after *in vitro* treatment with NVP. These volunteers were part of the research donor program at the Frederick National Laboratory that serves as a central source of fresh whole blood samples for *in vitro* research. To account for miR-148a dependent regulation of the *C*04:01* expression *HLA-C*06:02*, a high-expression allotype, was chosen as a control.

Treatment of lymphocytes with NVP for 24 hours *in vitro* did not result in increased expression of HLA-C on the cell surface of *C*04:01* or *C*06:02* positive volunteers, characterised by a positive peak shift to the right (figure 4.8).

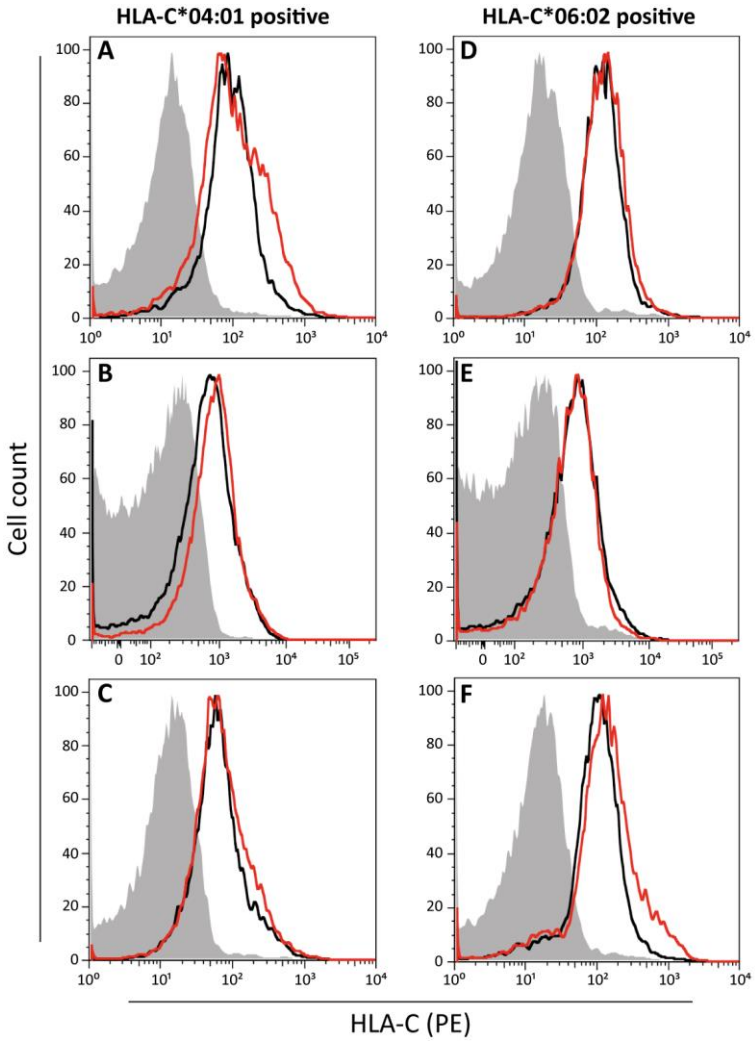


Fig. 4.8: HLA-C expression analysis using flow cytometry on lymphocytes from healthy volunteers. Isolated lymphocytes were treated with 10% DMSO or 25 µM NVP for 24 hours. Antigen expression was analysed with a primary mouse anti-human HLA-C specific antibody. The secondary PE-conjugated anti-mouse antibody is used to capture the surface expression or MFI. Histograms of *HLA-C*04:01* (**A** – **C**) and -*C*06:02* (**D** – **F**) homozygous volunteers are shown above. Unsorted lymphocytes are shown in light grey. Treatment with 25 µM NVP (red line) does not affect HLA-C expression of *C*04:01* and *C*06:02* positive lymphocytes as shown by the overlapping histograms with the DMSO control (black line).

4.4 Discussion

In our cohort of HIV-1 positive patients from Malawi, carriers of *HLA-C*04:01* have been shown to be at higher risk of NVP hypersensitivity [OR 2.64 (95% CI: 1.13, 6.18)] and particularly severe cutaneous adverse events such as SJS and TEN [OR 17.52 (95% CI: 3.31, 92.8); (CARR *et al.*, 2013)]. Unlike the activation of T cells by ABC-bound HLA-B*57:01 (ILLING *et al.*, 2012; NORCROSS *et al.*, 2012; OSTROV *et al.*, 2012), no causative function of the HLA-alleles associated with NVP hypersensitivity has been shown to date. Moreover, it is not known whether differences in HLA expression affect T cell function and thus the development and progression of drug hypersensitivity. Due to the limited sample availability and rare incidence (5%) of severe NVP hypersensitivity, variations in HLA-C protein expression levels could not be investigated in NVP-hypersensitive patients from Malawi. For that reason, expression levels of miR-148a, a miRNA known to regulate *HLA-C* gene expression by binding to the 3' UTR, was investigated in serum samples from NVP-treated patients. It was hypothesised that a decrease in miR-148a expression following the initiation of NVP or during the acute phase of the reaction would indicate a possible increase in HLA-C expression on the cell surface and therefore intensified antigen presentation and subsequent stimulation of the immune system.

Several studies have shown that many circulating miRNAs are derived from blood cells and tumour tissue (CHEN *et al.*, 2008b; TAYLOR and GERCEL-TAYLOR, 2008). These non-hematopoietically derived miRNAs persist in the circulation and are highly stable in urine, blood plasma and serum (SHARKEY *et al.*, 2012; WANG *et al.*, 2012b). In order to optimise the miRNA extraction, quantification and subsequent data analysis, differing RNA extraction methods and analysis procedures were optimised for our sample set. To assess the RNA extraction efficiency, a constant amount of serum and RNA were used for each individual sample. A synthetic miRNA was spiked into each sample to account for possible variations in the extraction efficiency and serve as an internal normalisation control.

Expression levels of miR-148a were not affected by the mean CD4+ T cell count and heat inactivation. Furthermore, expression did not differ between carriers

of *HLA-C*04:01* and carriers of other "inhibited" *HLA-C* alleles as shown by a linear regression model. The analysis of baseline and post-treatment samples showed a statistically significant increase in miR-148a expression in hypersensitive patients, but not in tolerant controls. In contrast, the subsequent miR-148a analysis of unpaired tolerant and hypersensitive patients at baseline or after the initiation of NVP treatment did not exhibit a statistically significant increase in the expression, although a slight tendency towards a higher miR-148a expression could be observed after treatment with NVP. An increased number of samples may be required to investigate the subtle differences in gene expression observed in our post-treatment samples.

Contrary to our expectations, we did not observe a decrease in miR-148a expression levels in tolerant nor hypersensitive patients post-treatment but a significant increase during the acute phase of hypersensitivity. It is not clear if this increase is of functional relevance in our patients. Besides the regulation of *HLA-C* expression, miR-148a is also known to downregulate *HLA-G* expression (MANASTER *et al.*, 2012) and has been implicated in hepatitis B associated hepatocellular carcinoma (YUAN *et al.*, 2012), colorectal (TAKAHASHI *et al.*, 2012; ZHANG *et al.*, 2011a) and ovarian cancer (ZHOU *et al.*, 2012). Xu and colleagues showed that miR-148a down-regulation is facilitated via hypermethylation of its promoter region by DNA-methyltransferase 1 in breast cancer cells, thus leading to increased tumour angiogenesis and cell proliferation (XU *et al.*, 2013). Functional targets of miR-148a include the genes encoding insulin-like growth factor 1 receptor (IGF-1R, XU *et al.*, 2013), B cell lymphoma 2 (Bcl-2, ZHANG *et al.*, 2011a) and calmodulin-dependent protein kinase (CaMII, LIU *et al.*, 2010). Whether the increase in miR-148a observed in our hypersensitive patients during the acute phase of reactions relates to the increase in inflammation and keratinocyte apoptosis observed in cutaneous adverse events requires further investigation.

A general effect of NVP treatment on the expression levels of miR-148a cannot be excluded, although no statistically significant difference was observed in our tolerant patients six weeks after treatment initiation, due to the small sample size available. In addition, HLA-C analysis of *HLA-C*04:01* and *HLA-C*06:02*

homozygous volunteers using flow cytometry showed that *in vitro* treatment with NVP did not lead to an increase in antigen expression. To test if the underlying HIV-1 infection would influence HLA-C expression, stable *in vitro* HIV-1 infections of healthy volunteer samples could be established; although, due to the low incidence of NVP hypersensitivity in the general population, a large number of volunteers may be needed to detect a possible association.

The conclusions that can be drawn from this study are limited by the extensive list of possible miR-148a targets and availability of patient samples. Yet, a statistically significant increase in miR-148a expression was observed in acute, NVP-hypersensitive patients from Malawi. Possible implications of these results would have to be investigated in a more extensive cohort of NVP-treated patients, but this study does provide the basis for further miRNA profiling from serum samples of drug-induced hypersensitivity patients.

Chapter 5

Analysis of circulatory miRNAs in hypersensitive and tolerant nevirapine-treated patients

Contents

Figures

Tables

5.1 Introduction

The application of miRNAs as diagnostic or prognostic biomarkers has been widely investigated, with the majority of studies focusing on cancer patients. MiRNA profiling, defined as the measurement and identification of a specific subset of miRNAs deregulated in a disease, has led to the discovery of unique patterns of deregulated miRNA expression, for example in breast cancer (reviewed in FARAZI *et al.*, 2011). In addition, the expression of single miRNA marker has been shown to be altered in cardiovascular (CREEMERS *et al.*, 2012), cancer (WITTMANN and JÄCK, 2010) and immune-mediated diseases (TOMANKOVA *et al.*, 2011). A small number of disease-associated miRNAs are summarised in table 5.1. Although miRNAs discovered to date have not yet been translated into clinical practice, miRNAs have the potential to improve the diagnosis and prognosis of patients as well as complementing existing diagnostic markers.

Well preserved in blood plasma and serum (MITCHELL *et al.*, 2008), and because they can be easily measured and quantified by PCR, circulating miRNAs have the potential to act as non-invasive biomarkers for various conditions. A promising example is miR-122, a highly specific liver miRNA that is released into the circulation upon damage to the liver. Clinical studies have shown that miR-122, as well as high mobility group box-1 (HMGB1) and necrosis keratin-18 (K18) accurately identified the development of acetaminophen-induced acute liver injury in patients with normal ALT levels, suggesting a stronger diagnostic effciency than provided by the current biomarker ALT (ANTOINE *et al.*, 2013; STARKEY LEWIS *et al.*, 2011). In addition, elevation of miR-122 has also been reported to be a more sensitive and specific indicator of cholestatic liver injury (SHIFENG *et al.*, 2013) and non-alcoholic fatty liver disease (CERMELLI *et al.*, 2011) than traditional markers of liver damage, such as ALT/AST levels.

As the investigation of miR-148a in our cohort of NVP-treated patients did not show a specific association between NVP hypersensitivity and miRNA expression levels, miRNA profiling of 84 miRNAs previously described in human serum was performed to identify a subset or individual miRNA(s) specifically deregulated in patients with NVP-induced cutaneous adverse events compared to tolerant controls. The aims of the study were to (i) identify new miRNAs up-

or down-regulated during the acute phase of NVP-induced cADRs in a subgroup of 24 patients recruited in Malawi and to (ii) validate the array results in a larger group of NVP-treated patients.

Table 5.1: Short overview of disease associated, deregulated miRNAs

microRNA	Implicated disease	Reference
Cancer		
miR-17-5p	Gastric cancer	(TSUJIURA *et al.*, 2010)
miR-146	Lung cancer	(RABINOWITS *et al.*, 2009)
miR-155	Diffuse large B-cell carcinoma	(LAWRIE *et al.*, 2008)
	Breast cancer	(ZHU *et al.*, 2009)
	Lung cancer	(RABINOWITS *et al.*, 2009)
	Ovarian cancer	(RESNICK *et al.*, 2009)
miR-500	Hepatocellular carcinoma	(YAMAMOTO *et al.*, 2009)
Immune-mediated diseases		
miR-146	Rheumatoid arthritis	(NAKASA *et al.*, 2008)
miR-146a	Psoriasis	(SONKOLY *et al.*, 2008)
	Rheumatoid arthritis	(PAULEY *et al.*, 2008)
miR-148a	Systemic lupus erythematosus	(PAN *et al.*, 2010)
miR-155	Rheumatoid arthritis	(STANCZYK *et al.*, 2008)
	Multiple sclerosis	(JUNKER *et al.*, 2009)
miR-192	Inflammatory bowel disease	(WU *et al.*, 2008)
miR-203	Rheumatoid arthritis	(STANCZYK *et al.*, 2011)
Adverse drug reactions		
miR-122	Acetaminophen-induced liver injury	(STARKEY LEWIS *et al.*, 2011)

5.2 Materials and methods

5.2.1 Patient population

Serum samples from 24 patients, who were part of the initial cohort recruited at the Queen Elizabeth Central Hospital in Blantyre (Malawi) (characterised in section 2.2.1), were chosen for the analysis using the miRNA array. The samples consisted of twelve NVP-hypersensitive patients who developed NVP-induced cutaneous and systemic adverse events within the first six weeks of ART initiation. Twelve tolerant patients were selected as controls and matched for age and gender. Samples were included based on the availability of both baseline (prior NVP treatment) and post-treatment samples (acute or week 6

samples for hypersensitive and tolerant patients respectively). Thus a total of 48 samples were included in the miRNA array cohort. These samples represented the 'discovery cohort'.

An extended set of serum samples (designated the 'expanded cohort'), which had been previously used in the analysis of miR-148a (detailed in section 4.2.1), contained 70 patients. Of these 28 were NVP-hypersensitive and 42 were tolerant controls. The 'expanded cohort' comprised a total of 91 baseline and post-treatment samples (figure 5.1). However, due to the limited sample availability, 30 samples (18 hypersensitive and 12 tolerant) overlapped between the 'discovery' and the 'expanded cohort'.

5.2.2 RNA extraction

Based on the results shown in the chapter 4.2, RNA was extracted from 200 µl of serum using the QIAGEN miRNA Serum & Plasma kit (QIAGEN, Germany) according to the manufacturer's instructions. In order to avoid bias, serum samples from hypersensitive samples and matched controls were extracted in mixed batches, which was maintained throughout the extraction and qPCR procedure described below. The synthetic cel-miR-39 was spiked in after the initial denaturation step with QIAzol. A fixed volume of eluted RNA was used for normalisation of samples.

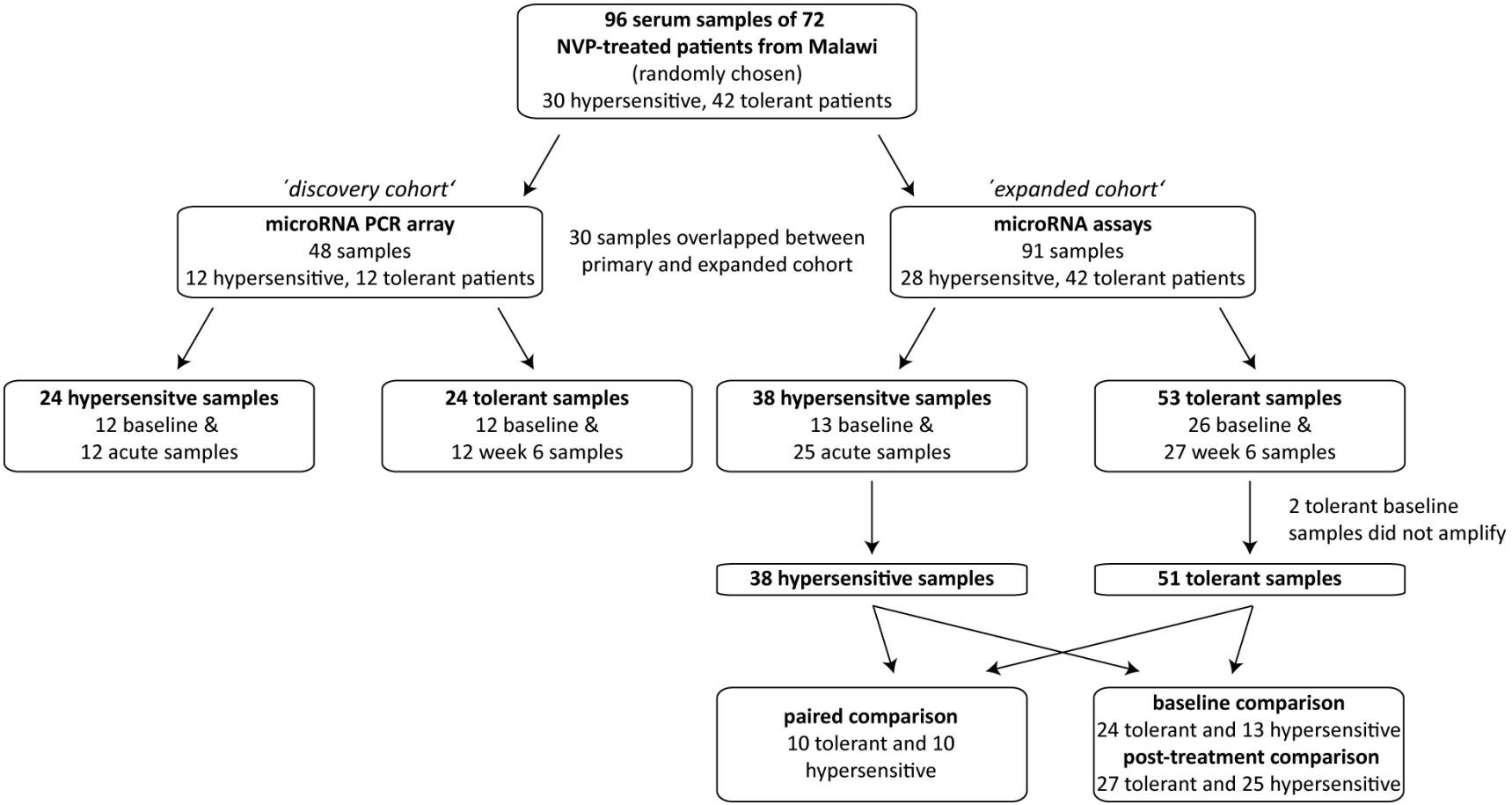


Fig. 5.1: Overview of samples used for miRNA profiling in NVP-treated patients from Malawi

5.2.3 Profiling of serum specific miRNAs in NVP-treated patients

MiRNA profiling using the miScript PCR system allows the detection and quantification of specific mature miRNAs. The human serum "pathway focused" array used in this study profiles the expression of 84 miRNAs (appendix 5.1). These miRNAs have been selected based on previously published results indicating the correlation of serum expression levels with various diseases (QIAGEN). Apart from the 84 miRNAs, a number of controls are also included in the array (table 5.2).

Table 5.2: Controls included in the miScript miRNA PCR Array

miRNA	Function
cel-miR-39	Data normalization; accounts for variances in extraction and amplification consistency
Mature miRNAs	miR-16, miR-21 and miR-191 Ubiquitously expressed miRNAs
snoRNAs/snRNA	SNORD61, SNORD68, SNORD75, SNORD95, SNORD96A and RNU6-B Data normalization using $2^{-\Delta\Delta C}{}_T$ method (not possible from serum samples)
miRTC	To asses the performance of reverse transcriptase
PPC	Assessment of PCR performance

snRNA small nuclear RNA, *snoRNA* small nucleolar RNA, *miRTC* miRNA reverse transcription control, *PPC* Positive PCR control

Reverse transcription for qPCR

Mature miRNAs as well as small nucleolar and small nuclear RNAs presented in the sample are specifically transcribed using the miScript HiSpec Buffer included in the miScript II RT kit (QIAGEN, Germany). While the transcription of mRNAs is suppressed, miRNAs are reverse transcribed using oligo-dT primers elongated by a universal tag following polyadenylation.

Template RNA was thawed on ice. For each sample RNA transcription mix containing 5x miScript HiSpec Buffer, 10x miScript Nucleics mix, 10.5 µl RNase free water, 2 µl miScript Reverse Transcriptase mix and 1.5 µl RNA were added to a 96-well plate. RNA was reverse transcribed on the Veriti® Thermal Cycler (Applied Biosystems, USA) under the following conditions: 60 min at 37°C and 5

min at 95°C. Each 20 µl reaction was diluted with 200 µl RNase free water and stored at -20°C until further use.

Expression profiling using miScript miRNA PCR arrays

Using the miScript miRNA PCR array in combination with the miScript SYBR Green PCR kit (QIAGEN, Germany) prepared cDNA is used as a template for the qPCR analysis. The miScript miRNA PCR array contains miRNA specific Primer Assays whereas the SYBR Green PCR kit contains the miScript Universal Reverse Primer. Each pathway-focused miScript PCR array allows the analysis of four samples simultaneously (see appendix 5.1 for a detailed plate layout).

Per sample a reaction mix was prepared containing 550 µl QuantiTect SYBR Green PCR master mix, 110 µl miScript Universal Primer, 340 µl RNase-free water and 100 µl template cDNA. Ten microliter of the reaction mix were added to each well on a miScript PCR array. The plate was sealed and centrifuged at 1000 g for one minute before the qPCR was run as follows: 5 min at 95°C and 40 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec and extension at 70°C for 30 sec.

Data analysis

Baseline and threshold values were defined for all PCR runs to allow comparison between different samples. The baseline was defined as the noise level at which no amplification was detected. On the other hand the threshold was set above the background in the lower half of the amplification plot. For all the experiments, the baseline was set between cycle 2 and 15 whereas the threshold was set at 0.15. A preliminary run of four samples showed good linearity between C_T values thus allowing us to perform further experiments without pre-amplification of miRNAs (data not shown).

The data was analysed using the online analysis tools provided by SABiosciences (SABIOSCIENCES) comparing the expression in tolerant and hypersensitive patients as well as between the different hypersensitivity phenotypes. The analysis software performs the following steps automatically:

a. all C_T values reported as not detectable or greater 35 cycles are changed to 35;
b. C_T values of PPC are evaluated. These should be 19 ± 2 cycles.
c. The C_T values of the miRTC are examined by calculating the ΔC_T as:

$$(Average\ miRTC\ C_T) - (average\ PPC\ C_T)$$

This value should be less than 7 to ensure no inhibition of the reverse transcription has occurred.

d. A ΔC_T value for each miRNA is calculated using the formula:

$$\Delta C_T = miRNA\ C_T - Average\ \textbf{cel-miR-39}\ C_T$$

Following, the fold change value for each miRNA is calculated as the $2^{-\Delta\Delta C_T}$:

$$\text{fold change} = 2^{-(\text{post-treatment } sample\ \Delta C_T - \text{baseline } sample\ \Delta C_T)}$$

e. Results were reported as "upregulation" if the fold change was greater than 1. In case the fold change was less than 1, the negative inverse was calculated and reported as "downregulation".

Inclusion criteria

MiRNAs with a C_T value < 35 cycles or that amplified in 75% or more samples were included in the final analysis. Of these, miRNAs with a fold change ≥ 2 or ≤ 2 were of further interest.

5.2.4 miR-205 detection using TaqMan® miRNA expression assays

Based on the miRNA array results reported below, miR-205 expression levels were further analysed in our 'expanded cohort'.

Reverse transcription and qPCR amplification

Performed by Vickie Marshall at the Frederick National Laboratory for Cancer Research (MD, USA) as part of a collaborative agreement.

Expression levels of miR-205, miR-16 and cel-miR-39 (table 5.3) were analysed as described before in section 4.2.3. Three microliters RNA were reverse

transcribed and 1.3 µl cDNA was added to each qPCR reaction. Samples were run in triplicates and NTCs for each miRNA were included as part of quality control.

Table 5.3: TaqMan® MicroRNA arrays

Assay Name	Assay ID	miRBase ID (version 20)	Mature miRNA sequence (5' – 3')
hsa-miR-16	000391	hsa-miR-16-5p	UAGCAGCACGUAAAUAUUGGCG
hsa-miR-205	000509	hsa-miR-205-5p	UCCUUCAUUCCACCGGAGUCUG
cel-mir-39	000200	cel-mir-39-3p	UCACCGGGUGUAAAUCAGCUUG

Provided by Applied Biosystems (USA).

Normalisation of C_T values

Using the SDS software (version 2.2; Applied Biosystems), C_T values were extracted and analysed. The median normalisation procedure proposed by Kroh *et al.* (2010) and McDonald *et al.* (2011) was used as described in chapter 4.2.4. After the normalisation, miRNA expression was calculated using the $2^{-\Delta C_T}$ as explained in section 2.2.5.

5.2.5 Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics software version 20 (IBM CORP., 2011).

Data analysis for miScript miRNA PCR array

Using a paired t-test, the analysis software provided by SABiosciences (SABIOSCIENCES) calculated p values for each miRNA between the control (baseline) and post-treatment group using the $2^{-\Delta C_T}$. Due to the large number of comparisons in tolerant and hypersensitive patients, the FDR was calculated for each miRNA included in the final analysis. Additionally, the fold change in miR-205 expression was compared between paired samples of tolerant, NIR and SJS patients.

Analysis of miR-205 expression levels using TaqMan® miRNA expression assays

The difference in expression levels was analysed using non-parametric tests, as data were not normally distributed. Expression levels of paired baseline and post-treatment samples were compared in tolerant and hypersensitive patients separately using a Wilcoxon test. At both baseline and post-treatment, the difference in expression between tolerant and different hypersensitivity phenotypes was tested using a Kruskal-Wallis test. In addition, the overall change in expression (calculated as the difference between paired baseline and post-treatment patient samples) was compared between tolerant and hypersensitive patients using a Mann-Whitney U test. A Bonferroni threshold of $p = 0.05/5 = 0.01$ (five tests) was deemed as statistically significant after correction for multiple testing.

5.3 Results

5.3.1 Population details

Demographic details of the 72 NVP-treated patients from Malawi are summarised in table 5.4. The majority of both tolerant (53%) and hypersensitive (60%) patients were female. Median age was 38 and 35 years for tolerant and hypersensitive patients, respectively. The mean body weight and CD4+ T cell count were 53.9 kg and 193 cells/µl in tolerant and 52.6 kg and 228 cells/µl in hypersensitive patients.

Of the 30 hypersensitive patients, 15 developed NIR (50%) and six HSS (20%). Nine patients (30%) were reported with severe cADRs, although one patient developed TEN and DILI simultaneously.

Table 5.4: Characteristics of NVP-treated patients included in this study

Covariate	Tolerant (n = 47)	Hypersensitive (n = 30)
Age, median years (IQR)		
	38 (33 – 41)	35 (34 – 42)
Gender, n (%)		
Male	22 (47)	12 (40)
Female	25 (53)	18 (60)
Body weight, kg (range)		
	53.9 (47.3 – 58.1)	52.6 (45.4 – 59.1)[a]
CD4+ T cell count (at NVP initiation), mean cells/µl (range)		
	193 (83 – 259)	228 (120 - 278)
Hypersensitivity reaction, n (%)		
NIR	-	15 (50)
HSS	-	6 (20)
SJS/TEN	-	9 (30)[b]

[a] body weight is missing for two hypersensitive patients. [b] one patient experienced both, SJS and DILI. *DILI* drug-induced liver injury, *IQR* interquartile range, *HSS* hypersensitivity syndrome, *NIR* nevirapine-induced rash, *NVP* nevirapine, *SJS* Stevens-Johnson syndrome

5.3.2 miRNAs differentially expressed in NVP-treated patients

The expression profile of 84 miRNAs (see appendix 5.1) was investigated in 12 NVP-hypersensitive and 12 tolerant patients ('discovery cohort'). Of the hypersensitive patients, seven experienced NIR, four SJS and one patient developed both TEN and DILI.

All samples passed the quality control check performed using the online analysis tool provided by SABiosciences (data not shown, SABIOSCIENCES). Eight of the 84 miRNAs were excluded, as their C_T values equalled 35 cycles. A further 31 miRNAs were excluded as less than 75% of the samples could be amplified. Of the 45 miRNAs included in the analysis, 21 miRNAs showed >2 (upregulation) or <-2 (downregulation) fold change in either tolerant controls, NVP-hypersensitive patients or both (figure 5.2).

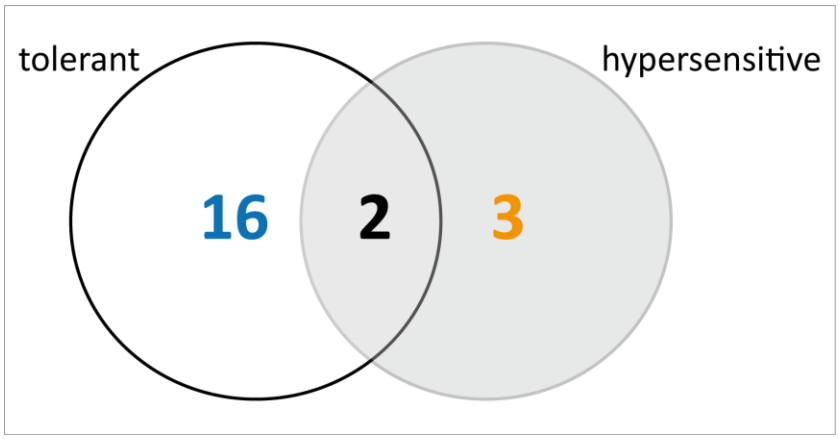


Fig. 5.2: Venn diagram depicting the distribution of deregulated miRNAs in NVP-tolerant and hypersensitive patients

Table 5.5 and figure 5.3 show the fold regulation of all 21 miRNAs in both, tolerant and hypersensitive patients. For completeness of data, the degree of regulation of all miRNAs is included, regardless whether the fold regulation was ≥ 2 or ≤ -2. Sixteen miRNAs were shown to be upregulated in tolerant patients after treatment with NVP. Of these, miR-122 and miR-27a were also shown to be upregulated in hypersensitive patients during the acute phase of the reaction, although in these patients the expression did not increase as strongly as observed in the tolerant controls (miR-122: 4.48 x and 2.37 x; miR-27a: 4.26 x and 2.09 x for tolerant and hypersensitive patients respectively). Expression of miR-124 showed a 3.2-fold downregulation in hypersensitive patients at the time of reaction ($p = 0.015$), but not six weeks after NVP treatment ($p = 0.391$). However, the association did not remain statistically significant after correction for multiple testing (FDR = 0.477). The expression of both miR-22 (2.1 x) and miR-205 (10.8 x) was upregulated in patients with HSRs to NVP.

Table 5.5: miRNAs differentially expressed in NVP-tolerant or hypersensitive patients

	Tolerant		Hypersensitive	
	Fold regulation	**P value†**	**Fold regulation**	**P value†**
miR-106b	**2.27**	0.262	-1.13	0.386
miR-122	**4.48**	0.054	**2.37**	0.895
miR-124	-1.10	0.391	**-3.22**	0.015
miR-146a	**3.58**	0.269	1.25	0.834
miR-148a	**2.97**	0.241	1.99	0.739
miR-15b	**2.10**	0.315	1.15	0.374
miR-16	**2.93**	0.244	1.31	0.274
miR-17-5p	**2.06**	0.289	1.09	0.390
miR-191	**2.46**	0.278	-1.66	0.885
miR-195	**3.37**	0.230	1.26	0.293
miR-19b	**2.64**	0.232	-1.12	0.421
miR-205	1.20	0.305	**10.79**	0.093
miR-21	**2.89**	0.249	1.88	0.126
miR-221	**2.22**	0.287	1.61	0.409
miR-22	1.23	0.325	**2.07**	0.159
miR-223	**4.35**	0.222	1.02	0.885
miR-23a	**2.84**	0.220	1.58	0.329
miR-25	**2.38**	0.255	1.53	0.277
miR-26a	**2.11**	0.306	-1.542	0.873
miR-27a	**4.26**	0.219	**2.09**	0.117
miR-30e	**3.14**	0.269	-1.10	0.596

21 miRNAs were differentially expressed in tolerant (blue), hypersensitive (yellow) or both (black). Respective tolerant or hypersensitive values have been included for comparison and are shown in grey if fold regulation did not reach ≥ 2 and ≤ -2. Statistical analysis was performed using a paired t-test. †FDR > 0.05 for all miRNAs.

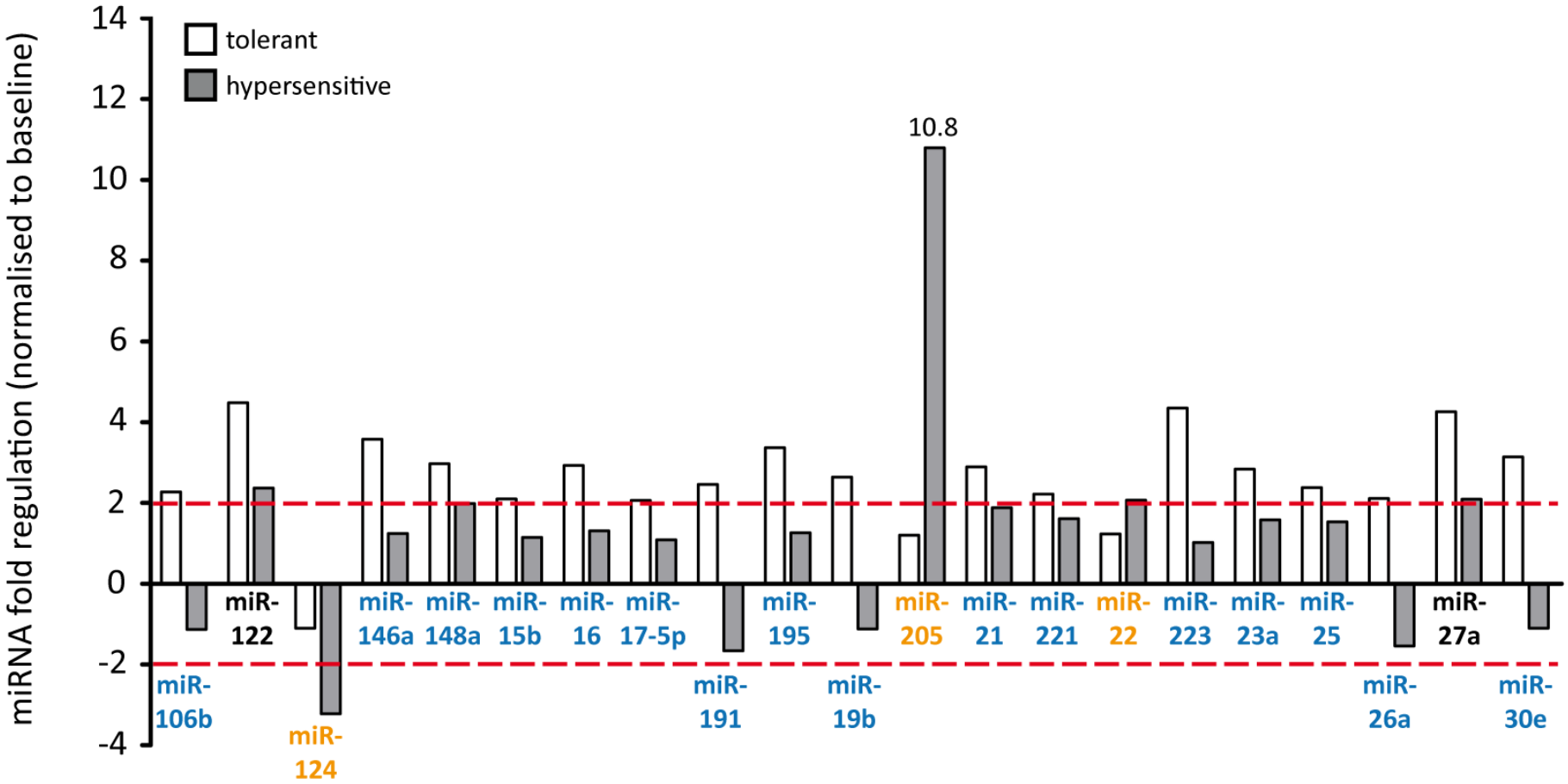


Fig. 5.3: miRNAs with a fold regulation > 2 or < -2 as well as corresponding tolerant or hypersensitive results

MiRNA expression analysis was performed between baseline and post-treatment samples of 12 tolerant controls (white bars) and 12 NVP-hypersensitive patients (grey bars). Fold change was calculated using the $2^{-\Delta\Delta C}{}_{T}$ method. Fold change values smaller than one were converted using the negative inverse. 21 miRNAs were shown to be differentially expressed; for comparison fold regulation of the respective tolerant or hypersensitive groups were included even if it did not reach ≥ 2 and ≤ -2. Statistical analysis was performed using a Student's t-test. None of the p values remained significant after calculating the FDR for each miRNA.

Based on the observation that miR-205 is the miRNA with the highest change in expression in hypersensitive patients, fold changes in NIR and SJS patients were analysed individually. The miR-205 expression levels of tolerant, NIR and SJS/TEN patients are shown in figure 5.4 below. There was higher miR-205 expression in cases than in controls (5.0 x and 31.5 x in NIR and SJS patients respectively), although these results were not significant because of the small patient numbers.

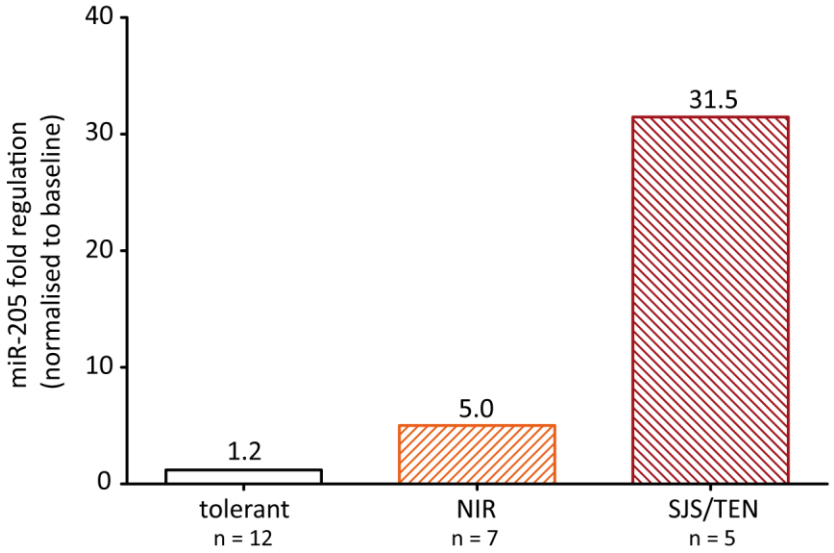


Fig. 5.4: Differences in miR-205 expression between tolerant controls and patients with NVP-induced rash or severe cutaneous adverse events.
Expression analysis was performed between baseline and post-treatment samples of tolerant (white bar), NIR (orange bar) and SJS/TEN patients (red bar). Using the $2^{-\Delta\Delta C}{}_{T}$ method the fold change was calculated and fold change values smaller than one were converted using the negative inverse. Statistical analysis was performed using a Student's t-test. None of the p values remained significant after correction for multiple testing. *NIR* nevirapine-induced rash, *SJS* Stevens-Johnson syndrome, *TEN* Toxic epidermal necrolysis

5.3.3 Detection of miR-205 expression using TaqMan® miRNA expression assays

To confirm the miScript miRNA array results, miR-205 expression levels were analysed in a cohort of 53 tolerant and 38 hypersensitive samples ('expanded cohort'). Eight of the hypersensitive patients developed HSS, 20 NIR and ten SJS. Samples taken from two tolerant patients at baseline failed amplification and were therefore excluded from all further analysis (see figure 5.1). Thus a total of

89 samples from 41 tolerant and 28 hypersensitive patients were included in this study.

The difference between baseline and post-treatment miR-205 expression was investigated in paired samples from ten tolerant or ten hypersensitive patients (figure 5.5 A) using a Wilcoxon test. Expression analysis via qPCR showed that miR-205 is significantly upregulated in hypersensitive patients during the acute phase of reaction (p = 0.007), but not in tolerant patients after six weeks of NVP treatment (p = 0.610). These results remained statistically significant after correction for multiple testing (Bonferroni threshold = 0.05/5 = 0.01).

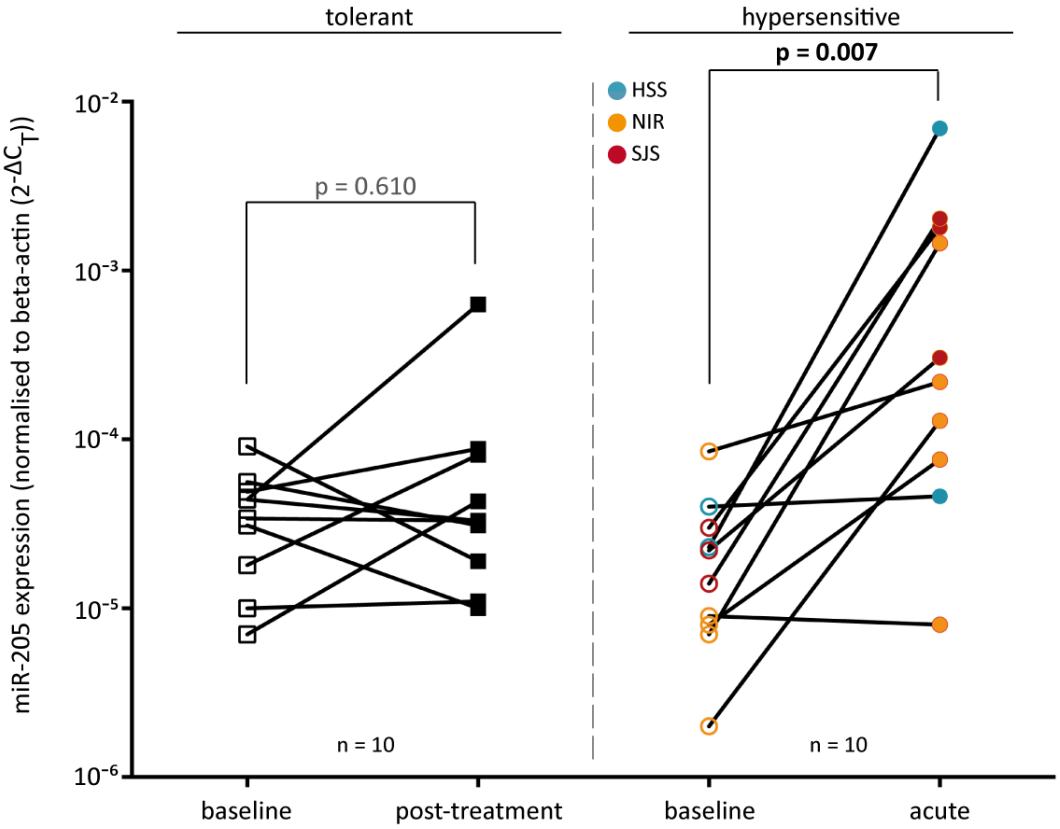


Fig. 5.5: Expression analysis of miR-205 in paired hypersensitive or tolerant samples
Wilcoxon test analysis of 10 paired tolerant control or NVP-hypersensitive samples. Post-treatment patient samples were taken six weeks after treatment initiation (tolerant) or during the acute phase of the reaction (hypersensitive). A significant difference could be detected between baseline and post-treatment samples from hypersensitive patients but not tolerant controls. Results remained significant after correction for multiple testing. HSS hypersensitivity syndrome, NIR nevirapine-induced rash, SJS Stevens-Johnson syndrome

In addition, the analysis of unpaired hypersensitive and tolerant patients showed a significant difference between the miR-205 levels of acute patients samples and tolerant controls six weeks after treatment initiation ($p < 0.001$; figure 5.6). At baseline, no statistically significant difference could be detected ($p = 0.083$).

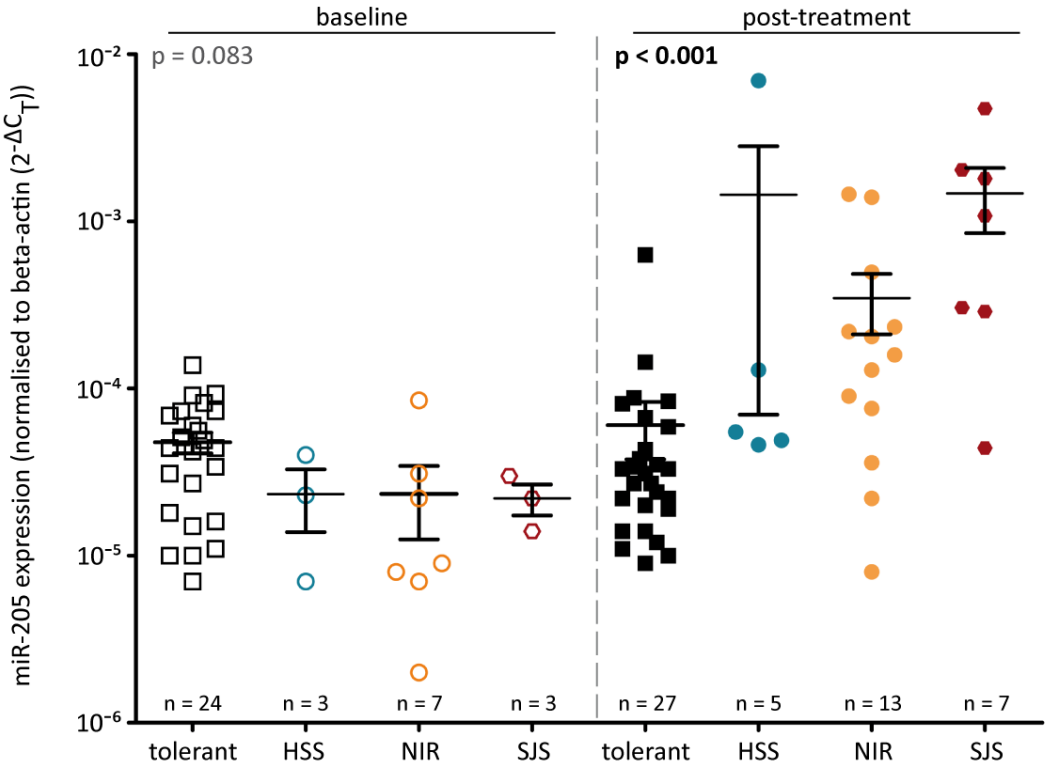


Fig. 5.6: Analysis of miR-205 expression at baseline or post-treatment

Differences between baseline (right panel) tolerant and hypersensitive samples as well as post-treatment samples (left panel) were analysed using a Kruskal-Wallis test. A significant difference in miR-205 expression could be detected between tolerant and hypersensitive patients after NVP treatment initiation. Results remained significant after Bonferroni correction. HSS hypersensitivity syndrome, NIR nevirapine-induced rash, SJS Stevens-Johnson syndrome

Likewise, the difference between post-treatment and baseline miR-205 expression levels was analysed between paired tolerant and hypersensitive samples ($n = 10$; figure 5.7). This comparison also showed a significant difference between tolerant and hypersensitive patients ($p = 0.004$) that remained significant after correction for multiple testing (significant threshold = 0.05/5 = 0.01).

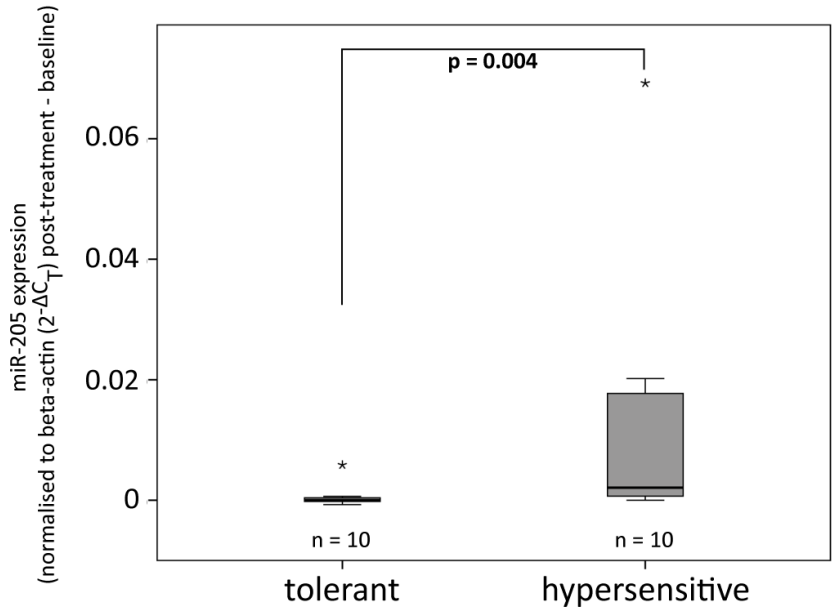


Fig. 5.7: Comparison of the difference between tolerant and hypersensitive patients
The difference in expression was calculated by subtracting the baseline ratio ($2^{-\Delta C_T}$) from the post-treatment ratio. A statistically significant difference was detected between tolerant and NVP-hypersensitive patients. Data was analysed using a Mann-Whitney U test and corrected for multiple testing using Bonferroni.

5.4 Discussion

Though a number of immunogenetic markers (MHC class I and II alleles) and polymorphisms in DME and drug transporters have been reported in association with NVP-induced HSRs across various populations (summarised in section 1.5.3), no diagnostic biomarker has been identified to date. Recently, methods allowing the quantification of non-coding RNA species through qPCR have been developed allowing researchers to focus on the diagnostic potential of miRNAs for the early detection and progression of various diseases. As yet no specific miRNAs have been identified as a reliable circulatory marker for drug-induced cADRs. Nevertheless miRNA profiling may lead to the identification of miRNAs that could potentially be used as biomarkers for the early detection and correct classification of either mild or severe cutaneous adverse events.

Therefore expression profiles of 84 serum miRNAs was investigated in samples from 12 NVP-hypersensitive patients and 12 tolerant controls. A total of 21 miRNAs were differentially expressed in either tolerant or hypersensitive samples after post-treatment expression was normalised to baseline expression

levels. However, none of the miRNAs differentially expressed in our 'discovery cohort' of NVP-treated patients reached statistical significance. This may be explained by the relatively small number of patients included in the analysis that may not be sufficient to control for the inter-individual variability in expression; however, due to the limited availability of paired patient samples in our cohort of black African patients, the number of hypersensitivity samples could not be increased. Although the use of a cDNA pre-amplification step might have improved the sensitivity of the array and therefore the number of detectable miRNAs, it might have also introduced a greater variability between the low abundance miRNAs and was thus not performed on our sample set. Nevertheless, we were able to detect 76 of the 82 miRNAs included in this array without the need of pre-amplification.

Of these miRNAs, miR-205 showed the highest increase in expression in NVP hypersensitivity patients (10.8 x), and specifically in NIR (5.0 x) and SJS/TEN patients (31.5 x) compared to tolerant controls (1.2 x). Therefore, we decided to further investigate the serum elevations of miR-205 in an expanded cohort of patients. The analysis of miR-205 in 89 serum samples from 69 NVP-treated patients confirmed our previous results that miR-205 expression levels are significantly upregulated during the acute phase of NVP-induced cADRs ($p = 0.007$), but not in tolerant patients after six weeks of NVP treatment ($p = 0.610$). In addition the analysis of tolerant and hypersensitive post-treatment samples showed a significant difference in miR-205 expression levels of tolerant, HSS, NIR and SJS patients ($p < 0.001$). No significant difference was observed between baseline samples of the different patient groups ($p = 0.083$). Albeit the average miR-205 threshold cycle ($C_T > 30$) was relatively high in both NVP-hypersensitive and tolerant patients, and thus results may have greater variability, we were able to detect a statistically significant difference in miR-205 expression between hypersensitive and tolerant patients. These findings suggest that circulatory miR-205 could either be used as a non-invasive biomarker for cADRs associated with NVP or may be implicated in the pathogenesis of severe cutaneous adverse events.

In order to assess the specificity of the elevated miR-205 levels for mild and/or severe skin reactions, additional work is needed to confirm if miR-205 is a specific marker of drug-induced cADRs. Incorporating a similar sized cohort of patients with NVP-induced hepatotoxicity into the analysis could be used to investigate whether elevated miR-205 levels are specific to cutaneous adverse events. In addition, other confounding factors, such as different skin conditions (e.g. psoriasis and dermatitis) and drugs, should be investigated. Furthermore, by measuring miR-205 levels in hypersensitive patients taking drugs known to cause cutaneous adverse events (e.g.: allopurinol, CBZ, lamotrigine, NSAIDs) and their respective tolerant controls, it could be determined if miR-205 is a unique marker of NVP hypersensitivity or general drug-induced cADRs. As our data was acquired from a homogenous Malawian population, it will have to be further established if the increase in miR-205 expression observed in our patients is subject to ethnic differences in other populations.

Next, studies investigating functional targets of miR-205 would prove useful to resolve the mechanistic role of miR-205 in cutaneous adverse events. An initial literature review indicated that miR-205 is highly expressed in keratinocytes (McKenna *et al.*, 2010) and part of the post-transcriptional regulation of keratinocyte migration (Yu *et al.*, 2010). MiR-205 was found to be expressed in epithelial cells, where it regulates SH_2-containing phosphoinositide 5'-phosphatase 2 (SHIP2) levels (Yu *et al.*, 2008). Cellular changes occurring after initial keratinocyte injury may lead to excess miR-205 shedding; the decrease in miR-205 levels could then lead to an increase in SHIP2 expression, thus leading to an increase keratinocyte apoptosis. Further, miR-205 has been shown to target Bcl-w (Bhatnagar *et al.*, 2010), a member of the Bcl-2 family known to promote cell survival. The implications of deregulated miR-205 levels in keratinocytes could be investigated using the techniques proposed by Ichihara *et al.* (2013) (Ichihara *et al.*, 2013): using *in situ* hybridization techniques, the cellular localisation of miR-205 could be determined in skin biopsies of NVP-hypersensitive patients at the time of reaction. Next, the role of miR-205 in keratinocyte apoptosis could be investigated *in vitro*, through transfection of miR-205 mimics into an immortalised human keratinocyte cell line. These experiments would also allow testing the effect of NVP and other drugs

associated with severe cADRs on keratinocyte apoptosis in correlation with miR-205 levels. Finally, potential targets of miR-205 could be identified using miRNA target prediction databases (e.g. www.targetscan.org) and validated by luciferase reporter assays *in vitro*.

In addition, a principal component analysis or similar methods could be used to define a specific set of miRNAs differentially expressed between hypersensitive patients and tolerant controls (TAGUCHI and MURAKAMI, 2013). This type of analysis has been already utilised to compose miRNA-panels differentiating sera from breast cancer patients and healthy volunteers (CHAN *et al.*, 2013). Thus a disease-specific miRNA signature might be used to distinguish between patients that are at risk of developing NVP hypersensitivity or not.

This study identified an increase in miR-205 expression levels in serum samples from NVP-hypersensitive patients from Malawi during the acute phase of the reaction. This was further validated in an extended cohort of NVP-treated patients. Further studies are needed to establish how miR-205 contributes to the pathogenesis of severe NVP-induced cADRs and whether serum miR-205 levels could be used as a circulatory marker for these reactions.

Chapter 6

HLA-associations with nevirapine-induced hypersensitivity: a systematic review of the literature and meta-analysis

Contents

Figures

Tables

6.1 Introduction

Nevirapine is frequently prescribed as part of HAART. However, drug hypersensitivity, comprising a number of manifestations ranging from NVP-induced rash to severe blistering skin reactions (such as SJS and TEN) and hepatotoxicity, occurs in about 5% of NVP-treated patients within the first few weeks of therapy. Several features of NVP hypersensitivity, including the delay between treatment initiation and onset of reactions, the faster development of symptoms after rechallenge and the inflammatory cell infiltrate in lesions, suggest that the reactions may be immune-mediated. In addition, genetic variations may affect the risk of ADRs associated with NVP treatment. This would be consistent with other drugs; for example, numerous HLA-alleles have been described as susceptibility markers for HSRs to ABC (HETHERINGTON *et al.*, 2001; MALLAL *et al.*, 2002), CBZ (CHUNG *et al.*, 2004; MCCORMACK *et al.*, 2011) and allopurinol (HUNG *et al.*, 2005).

Since 2005, when the first association between *HLA-DRB*01:01* and NVP-induced hepatotoxicity was reported (MARTIN *et al.*, 2005), several other MHC class I and class II alleles have been identified as risk factors for NVP-induced adverse effects: *HLA-B*35* [Caucasians (PHILLIPS *et al.*, 2011), Indians (UMAPATHY *et al.*, 2011) and Thai (CHANTARANGSU *et al.*, 2009; YUAN *et al.*, 2011)], *HLA-B*58:01* [mixed population (PHILLIPS *et al.*, 2013)], *HLA-C*04* [black Africans (CARR *et al.*, 2013; YUAN *et al.*, 2011), Caucasians (YUAN *et al.*, 2011), Han Chinese (GAO *et al.*, 2012) and Thai (LIKANONSAKUL *et al.*, 2009)], *HLA-C*08* [Caucasians (LITTERA *et al.*, 2006) and Japanese (GATANAGA *et al.*, 2007)] and various *HLA-DRB1*01* allelotypes [whites (PHILLIPS *et al.*, 2013; PHILLIPS *et al.*, 2011; VITEZICA *et al.*, 2008; YUAN *et al.*, 2011)] (summarised in table 1.8).

Given the number and diversity of HLA-alleles reported in relation to NVP-induced HSRs, the aims of this chapter were (i) to perform a systematic literature search to identify all studies investigating HLA-alleles and NVP hypersensitivity and (ii) to summarise the results from multiple studies and perform a meta-analysis to improve the precision and accuracy of estimates.

6.2 Methods

Search strategy

The following databases and web sources were searched in April 2013 applying the search strategy summarised in table 6.1: MEDLINE via Pubmed, ISI Web of Science, Cochrane Database of Systematic Reviews (CDSR), Cochrane Central Register of Controlled Trials (CENTRAL), Health and Technology Assessment (HTA) database, *meta*Register of Controlled Trials (mRCT) and www.clinicaltrials.org. No date restrictions were applied to the search strategy as studies evaluating the association of HLA-alleles and NVP-induced hypersensitivity reactions have only started to emerge since 2005. Bibliographies of published studies and reviews were also screened for additional references. The quality of our search strategy was assessed by comparison with a list of previously identified publications (CARR *et al.*, 2013). A preliminary search scanning all titles for eligibility was performed with any irrelevant studies removed from further analysis. The abstracts of potentially qualifying studies were then screened and the full text articles obtained for those identified as potentially meeting the inclusion criteria. This process was performed by myself. Finally, the full text articles were reviewed and assessed for inclusion. The final number of included studies was agreed upon after discussion with Dr. Dan Carr.

Table 6.1: Search strategy

#	Search term
1	Nevirapine or NVP or antiretroviral
2	"Human leukocyte antigen" or "human leukocyte antigens" or HLA
3	ADR or "adverse drug reaction" or "adverse drug reactions" or "adverse event" or "adverse events"
4	"Drug effect" or "drug effects" or "drug toxicity"
5	HSR or hypersensitivity or "hypersensitivity reaction" or "hypersensitivity reactions"
6	#1 and #2 and (#3 or #4 or #5)

Eligibility criteria

The study selection process is outlined in figure 6.1. Prospective and retrospective case-control and cohort studies, as well as clinical trials investigating HLA genotype associations and NVP hypersensitivity were further evaluated. No geographical limitations were applied and any genotyping technique was eligible. However, the analysis was restricted to the English-language literature and publications that did not include original research, such as reviews and case reports, were excluded from the review. In addition, research groups were contacted for information if data on carrier frequency and HLA associations were not available. Studies that compared population controls against NVP-hypersensitive patients instead of tolerant controls were removed from the meta-analysis but included in the final review.

Data extraction

Data extraction was conducted using a standardised data extraction sheet, which was tested on the first three studies. This included data on study design, location of study, ethnicity of populations, HLA genotyping, HLA-alleles analysed, number of cases and controls (overall and for each genotype), type and definition of adverse events. Quality of included studies was assessed in agreement with the criteria laid out by Jorgensen and Wiliamson for the quality control of pharmacogenetic studies (COBOS *et al.*, 2011; JORGENSEN and WILLIAMSON, 2008).

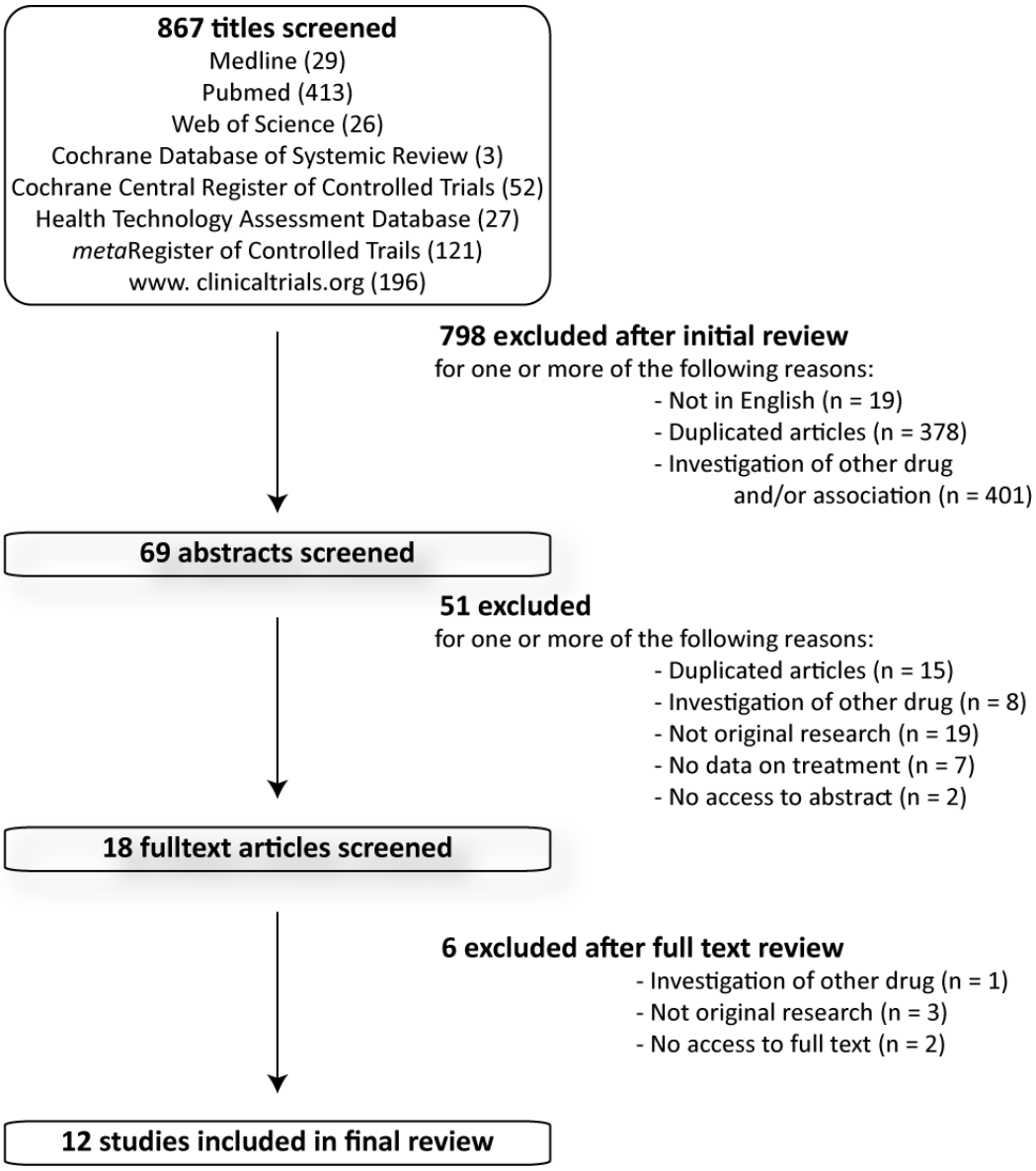


Fig. 6.1: Flowchart of the literature review progress

Data analysis

Studies were stratified by both HLA-allele and associated adverse reactions. HLA-alleles were combined as low or high resolution alleles. The phenotype classification used in the different studies varied widely. In order to attempt to standardise this, the reactions were first classified as cADRs (NIR, HSS, SJS/TEN), where HSS implied systemic involvement (for example of the liver). Some studies also evaluated patients with hepatotoxicity, which may have occurred in isolation or as part of a hypersensitivity syndrome. These were

further grouped as general hypersensitivity if both cutaneous and hepatic events were included in the analysis.

The reported populations were subdivided into black Africans (South Africans, Malawians and Africans), Caucasians (French, Western Australia and European), North-East Asians (Taiwanese and Han Chinese) and Thai. Studies reporting predominantly white and black patient populations were included in the analysis of Caucasians and black Africans, respectively.

All statistical analyses were undertaken using the Review Manager [RevMan version 5.2 (THE NORDIC COCHRANE CENTRE, 2012)] and IBM SPSS Statistics programs [version 20 (IBM CORP., 2011)]. Odds ratios and corresponding 95% CIs were calculated for each study. Using the Mantel-Haenszel random effects model, a summary estimate (overall OR) was calculated to determine the effect of a particular HLA-allele on the risk of NVP-induced ADRs. Forest plots, created by RevMan, were inspected and statistical heterogeneity was evaluated using the I^2 statistic, which describes the percentage of variability across studies that is due to real differences and not chance. Heterogeneity was classified according to the Cochrane Handbook for Systematic Reviews of Interventions (HIGGINS and SALLY, 2011): 0-40% may not be important, 30-60% represents moderate heterogeneity, 50-90% may describe substantial and 75-100% suggests considerable heterogeneity. If significant heterogeneity was observed, possible reasons were considered (e.g. ethnicity, definition of HSRs, etc.). Each analysis had to include at least one population with two or more studies. Subgroup analysis stratified by ethnic group was performed if associations had been described in more than one population.

6.3 Results

Our initial search identified 867 publications that reported original data on the association of HLA-alleles and adverse reactions to NVP (see also figure 6.1). After screening the titles, 789 studies were excluded; a further 51 were excluded after scanning the abstracts and a further 6 were excluded after

reviewing the full study reports. This left twelve studies to be included in our final review (see table 6.2; for a more extensive table refer to appendix 2).

The study conducted by Vitezica *et al.* (2008) investigated the association of *HLA-DRB1*01* and cADRs in a cohort that included 14 NVP- and seven EFV-hypersensitive patients. However, it was not possible to distinguish between the EFV- and NVP-treated patients in the analysis. Based on the findings that NVP-treated patients are more likely to develop skin toxicities compared to EFV (SHUBBER *et al.*, 2013), we concluded that the effect observed in this study is likely driven by NVP treatment alone.

6.3.1 Characteristics of included studies

Twelve case-control studies were included in this report (characteristics are summarised in table 6.2). A total of 2362 NVP-treated patients were genotyped, of which 773 were classified as hypersensitive patients and 1513 as tolerant controls.

Sample size

The median sample size was 101 (IQR: 55 - 262) and only one study performed a sample size calculation prior to analysis (CARR *et al.*, 2013).

Hardy-Weinberg Equilibrium

Only two studies reported testing for HWE (CARR *et al.*, 2013; YUAN *et al.*, 2011), whereas ten did not mention undertaking any tests of whether the observed genotypes deviated from HWE.

Table 6.2: Characteristics of case-control studies included in our final review

Study	Country	Ethnicity	Assigned phenotype	Phenotype definition	HLA-alleles analysed	HLA genotyping	Typing method	Reported HLA risk alleles	Sample size	# of cases	Reported OR [95% CI]
(CARR *et al.*, 2013)	Malawi	Black African	cADRs, hepatotoxicity or general hypersensitivity	Maculopapular rash without systemic manifestations (NIR); rash with blistering eruptions and involvement of 2 mucous membranes (SJS/TEN); Widespread rash and systematic manifestations (HSS); Visible jaundice and abnormal ALT levels (DILI)	*HLA-A, -B, -C, -DRB1, -DQB*	High resolution	SBT	*HLA-C*04:01*	271	117	2.84 [1.13, 6.18] for overall HSRs; 17.52 [3.31, 92.8] for SJS/TEN
(CHANTARANGSU *et al.*, 2009)	Thailand	Thai	cADRs	Skin rash (according to the NIAID Division of AIDS criteria)	*HLA-A, -B, -C, -DPB1, -DQB1, -DRB1*	High resolution	SBT	*HLA-B*35:05*	335	147	18.96 [4.87, 73.44]
(GAO *et al.*, 2012)	China	Han Chinese	General hypersensitivity	Extensive skin rash, bullous skin lesions or skin manifestations combined with fever and/or hepatotoxicity with elevated ALT levels (> 5 ULN) (according to the AIDS clinical trial group grading severity list)	*HLA-C, -DRB1*	Low resolution	PCR-SSP	*HLA-C*04*	103	32	3.61 [1.14, 11.49]

Table 6.2: continued

Study	Country	Ethnicity	Assigned phenotype	Phenotype definition	HLA-alleles analysed	HLA genotyping	Typing method	Reported HLA risk alleles	Sample size	# of cases	Reported OR [95% CI]
(GATANAGA *et al.*, 2007) †	Japan	Mixed (96% Japanese, 2% Korean, 2% White American)	General hypersensitivity	Extensive skin rash with or without fever; hepatotoxicity with elevated AST/ALT levels >3 ULN	*HLA-C*	Low resolution	PCR-SSP	*HLA-C*08*	41	12	NA
(GOZALO *et al.*, 2011)	France	Mixed (majority of patients Caucasian)	General hypersensitivity	Cutaneous (grade 3 or 4) and hepatic toxicity (ALT > 3 ULN), not always linked	*HLA-DRB1*	High resolution	PCR-SSO	*HLA-DRB1*01:01*	71	11	NA
(LIKANONSAKUL *et al.*, 2009)	Thailand	Thai	cADRs	Skin rash without hepatotoxicity	*HLA-C*	Low resolution	PCR-SSP	*HLA-C*04*	99	39	NA
(LITTERA *et al.*, 2006) †	Italy	Sardinian	General hypersensitivity	Extensive skin rash, bullous or scaling skin lesions combined with fever, myalgia, arthralgia and/or liver toxicity (≥ grade 3 and AST/ALT ≥ 5 ULN	*HLA-A, -B, -C, -DR*	Low resolution	PCR-SSP	*HLA-C*08*	49	13	NA
(MARTIN *et al.*, 2005)	Australia	Mixed (83% Caucasian)	Hepatotoxicity	Drug-induced rash with hepatotoxicity (grade 2) and/or fever	*HLA-A, -B, -C, -DQ, -DR*	High resolution	Serological & SBT or PCR-SSP	*HLA-DRB1*01:01*	235	14	17.70 [NA]
(PHILLIPS *et al.*, 2013)	South Africa	Mixed (71% Black, 14% Caucasian, 11% mixed, 2% Asians, 1% other)	Hepatotoxicity	Hepatotoxicity [grade 3 (ALT/AST > 5x ULN) or 4 (ALT/ST > 10x ULN)] with or without associated symptoms	*HLA-A, -B, -C, -DRB1*	High resolution	SBT	*HLA-B58:01* *HLA-DRB1*01:02*	168	57	NA

Table 6.2: continued

Study	Country	Ethnicity	Assigned phenotype	Phenotype definition	HLA-alleles analysed	HLA genotyping	Typing method	Reported HLA risk alleles	Sample size	# of cases	Reported OR [95% CI]
(UMAPATHY *et al.*, 2011) †	India	Indian	cADRs	Extensive skin rash with or without hepatitis and fever	*HLA-B*	Low resolution	Serological	*HLA-B*35*	80	40	3.38 [1.54, 7.41]
(VITEZICA *et al.*, 2008)	France	Caucasian	cADRs	Skin rash	*HLA-DRB1*	Low resolution	PCR-SSP	*HLA-DRB1*01*	21	6	NA
(YUAN *et al.*, 2011)	Argentina, Australia, Canada, France, Germany, Netherlands, Spain, Taiwan, Thailand, UK, USA	Asian, black, Caucasian, Thai	cADRs or hepatotoxicity	Severe cutaneous toxicity (grade 3 or 4 according to the NIAID Division of AIDS criteria) Symptomatic hepatic AST/ALT elevations (≥ 5x ULN) or acute liver failure	*HLA-A, -B, -C, -DPB, -DQB, -DRB*	Low resolution	PCR-SSO	*HLA-B*35* *HLA-C*04* *HLA-DRB1*01*	889	276	NA

† denotes studies that were not included in the meta-analysis but will be discussed as part of our review. *cADRs* cutaneous adverse drug reactions, *CI* confidence interval, *HLA* human leukocyte antigen, *NA* not available, *OR* odds ratio, *SBT* Sequence Based Typing, *PCR-SSP or SSO* polymerase chain reaction with sequence-specific primer or oligonucleotides, *ULN* upper limit of normal

HLA genotyping

Eight studies investigated both MHC class I and class II alleles (CARR *et al.*, 2013; CHANTARANGSU *et al.*, 2009; GAO *et al.*, 2012; GATANAGA *et al.*, 2007; LITTERA *et al.*, 2006; MARTIN *et al.*, 2005; PHILLIPS *et al.*, 2013; YUAN *et al.*, 2011), while four studies genotyped specific alleles based on previously published reports (GOZALO *et al.*, 2011; LIKANONSAKUL *et al.*, 2009; UMAPATHY *et al.*, 2011; VITEZICA *et al.*, 2008). Only a minority of studies report quality checks for genotyping [three out of twelve studies (GOZALO *et al.*, 2011; LIKANONSAKUL *et al.*, 2009; YUAN *et al.*, 2011)].

HLA-alleles were serologically or molecularly typed [see table 6.2; low (e.g. *C*04*) versus high resolution (e.g. *C*04:01*)]. Six studies investigated associations of HLA-alleles to NVP hypersensitivity using low resolution typing methods, which cannot identify individual alleles but broad families of HLA genotypes. If high resolution molecular typing of HLA was used, all alleles belonging to one group were merged so that genotyping data could be pooled across different reports.

Population and outcome definitions

Of the five studies investigating associations in mixed populations (GATANAGA *et al.*, 2007; GOZALO *et al.*, 2011; MARTIN *et al.*, 2005; PHILLIPS *et al.*, 2013; YUAN *et al.*, 2011), four studies classified the population according to the ethnicity of the majority of patients, whereas the study by Yuan *et al.* defined different subpopulations in which subgroup specific analyses were performed. Only one study recruited patients in more than one country (YUAN *et al.*, 2011). Two studies included NVP-tolerant patients as well as population controls (CHANTARANGSU *et al.*, 2009; LITTERA *et al.*, 2006).

Four studies recruited NVP-hypersensitive patients who developed cADRs only (CHANTARANGSU *et al.*, 2009; LIKANONSAKUL *et al.*, 2009; UMAPATHY *et al.*, 2011; VITEZICA *et al.*, 2008). Two studies performed subgroup analyses of cADRs and hepatic events for specific associations (CARR *et al.*, 2013; YUAN *et al.*, 2011) while multisystem reactions were analysed in five studies (GAO *et al.*, 2012;

GATANAGA *et al.*, 2007; GOZALO *et al.*, 2011; LITTERA *et al.*, 2006; MARTIN *et al.*, 2005). Only one study investigated HLA associations with isolated hepatotoxicity (PHILLIPS *et al.*, 2013).

Outcome definitions differed among all twelve studies. Skin rash was classified according to previously published guidelines [Division of AIDS Table for Grading the Severity of Adult and Paediatric Adverse Events (DIVISION OF AIDS)] in three studies (CHANTARANGSU *et al.*, 2009; GAO *et al.*, 2012; YUAN *et al.*, 2011). Predefined, albeit more generalised terms were used by nine studies for the definition of NVP-induced HSRs (CARR *et al.*, 2013; GATANAGA *et al.*, 2007; GOZALO *et al.*, 2011; LIKANONSAKUL *et al.*, 2009; LITTERA *et al.*, 2006; MARTIN *et al.*, 2005; PHILLIPS *et al.*, 2013; UMAPATHY *et al.*, 2011; VITEZICA *et al.*, 2008). Among these, hepatic reactions were categorised based on elevated liver function tests, but different thresholds for AST/ALT elevations were used in each study (CARR *et al.*, 2013; GAO *et al.*, 2012; GATANAGA *et al.*, 2007; GOZALO *et al.*, 2011; LITTERA *et al.*, 2006; MARTIN *et al.*, 2005; PHILLIPS *et al.*, 2013; YUAN *et al.*, 2011).

6.3.2 Meta-analyses

Associations of HLA-B with nevirapine-induced hepatic and cutaneous adverse events

Data on the association between *HLA-B*58:01* and NVP-induced hepatotoxicity in black African patients was available in two studies [see figure 6.2 (CARR *et al.*, 2013; PHILLIPS *et al.*, 2013)]. There was a significant difference in the risk of hepatotoxicity in NVP-hypersensitive patients carrying the *B*58:01* allele and there was no heterogeneity between the studies [OR 3.51 (95% CI: 1.72, 7.19); I^2 0%].

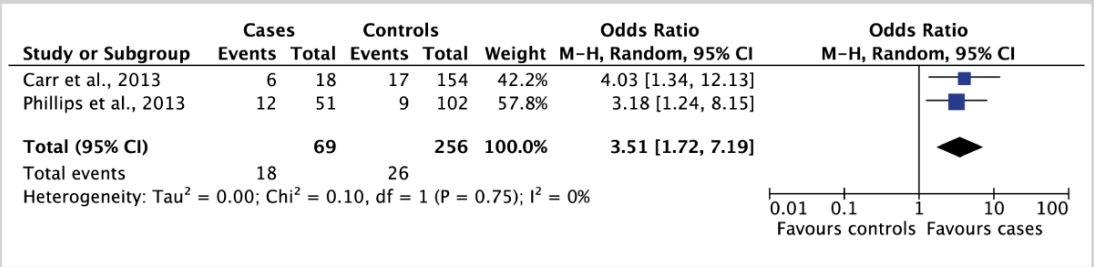


Fig. 6.2: Forest plot for the association between *HLA-B*58:01* and hepatotoxicity in black Africans

Data analysed using the Mantel-Haenszel method. Carriers are reported as events. Horizontal lines depict 95% CI and squares are proportional to sample size. Diamonds represent pooled ORs. Heterogeneity is measured by I^2. *CI* confidence interval, *HLA* human leukocyte antigen

Three studies investigated the association between *HLA-B*35* and NVP-induced cADRs in four different populations (CARR *et al.*, 2013; CHANTARANGSU *et al.*, 2009; YUAN *et al.*, 2011): Black Africans, Caucasians, North-East Asians and Thai (presented in figure 6.3). The pooled effect estimate was statistically significant [OR 2.45 (95% CI: 1.10, 5.48)], although there was substantial heterogeneity amongst the populations (I^2: 69%). The association was largely driven by the Thai population, where the effect estimate was highly significant [OR 9.34 (95% CI: 2.68, 32. 58); I^2 51%] and moderate heterogeneity was evident in this subpopulation. Carriers of *HLA-B*35* of black African [OR 0.97 (95% CI: 0.40, 2.37); I^2 0%], Caucasian [OR 1.79 (95% CI: 0.99, 3.23)] and North-East Asian [OR 1.53 (95% CI: 0.40, 5.82)] origin did not show a significant increase in the likelihood of cADRs.

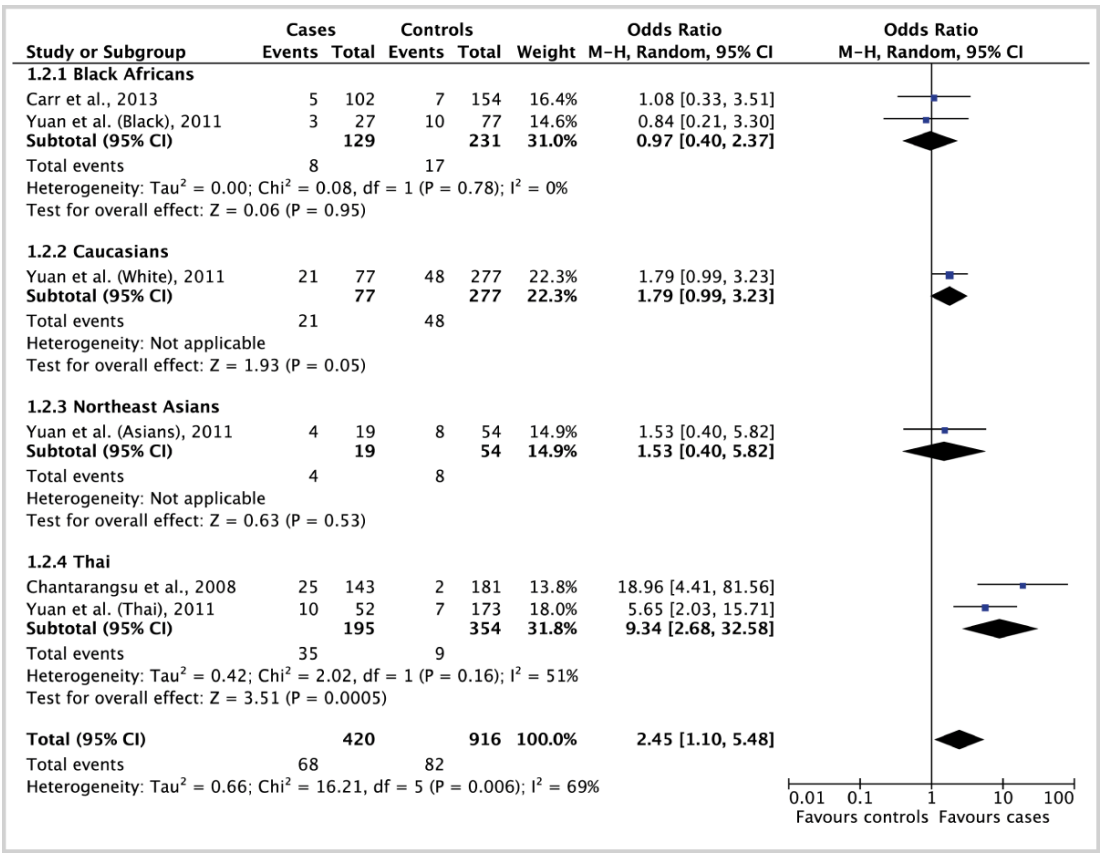


Fig. 6.3: Association between *HLA-B*35* and NVP-induced cutaneous adverse reactions

Forest plot for the analysis of *HLA-B*35* and cADRs using the Mantel-Haenszel method. Events describe the number of carriers. Size of square is proportional to sample size. Horizontal lines represent 95% CI while diamonds indicate pooled ORs. I^2 is a measure of heterogeneity. *cADRs* cutaneous adverse drug reactions, *CI* confidence interval, *HLA* human leukocyte antigen

*HLA-C*04 and hypersensitivity reactions in nevirapine-treated patients*

Three studies investigated the association of *HLA-C*04* and NVP-induced cutaneous adverse reactions in black African, Caucasian, North-East Asian and Thai patients (CARR *et al.*, 2013; LIKANONSAKUL *et al.*, 2009; YUAN *et al.*, 2011). The overall pooled effect was significant [OR 2.63 (95% CI: 1.97, 3.52); I^2 0%] with no heterogeneity between studies (shown in figure 6.4). Effect estimates were significant for black Africans [OR 3.36 (95% CI: 1.99, 5.65); I^2 14%], Caucasians [OR 1.92 (95% CI: 1.11, 3.35)] and Thai [OR 2.56 (95% CI: 1.46, 4.49); I^2 0%], but not for North-East Asians [OR 2.92 (95% CI: 0.90, 9.45)], presumably because of the small sample size for the latter.

The association between *HLA-C*04* and all phenotypes of NVP-induced hypersensitivity was examined in five studies (CARR *et al.*, 2013; GAO *et al.*, 2012;

LIKANONSAKUL *et al.*, 2009; YUAN *et al.*, 2011), but did not affect the overall estimate significantly [data not shown; OR 2.59 (95% CI: 1.96, 3.43); I^2 0%].

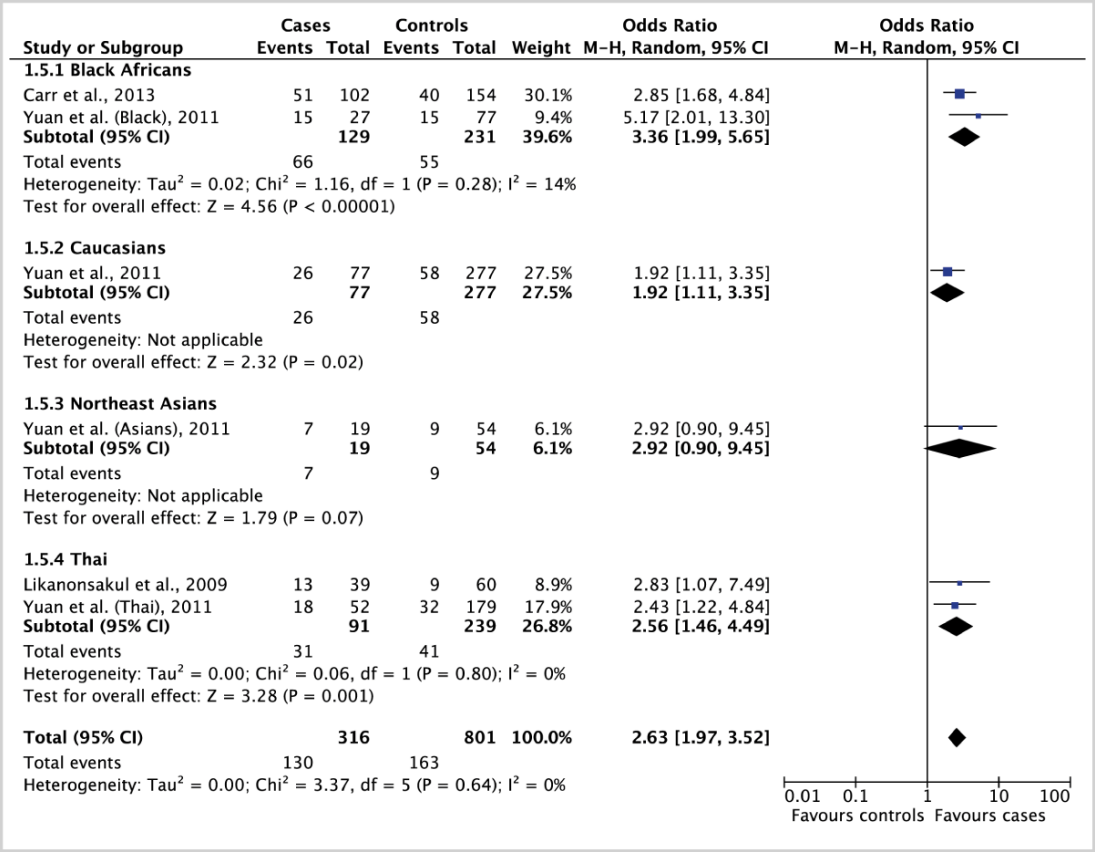


Fig. 6.4: Forest plot for the analysis of NVP-induced cADRs and *HLA-C*04*
Analysis of *HLA-C*04* in NVP-induced. Data analysis was performed using the Mantel-Haenszel test. Carriers are reported as events. Different sizes of squares indicate the sample size and horizontal lines represent 95% CI. Diamonds describe the pooled OR. I^2 is a measure of heterogeneity. *cADRs* cutaneous adverse drug reactions, *CI* confidence interval, *HLA* human leukocyte antigen

*HLA-DRB1*01 alleles and hypersensitivity reactions associated with nevirapine*

Associations between *HLA-DRB1*01* and NVP-induced hepatotoxicity have been tested in four studies (CARR *et al.*, 2013; MARTIN *et al.*, 2005; PHILLIPS *et al.*, 2013; YUAN *et al.*, 2011). Again, the pooled effect estimate was significant [figure 6.5; OR 2.94 (95% CI: 1.92; 4.0); I^2 0%]. For the black African [OR 2.14 (95% CI: 1.02, 4.50); I^2 0%] and Caucasian [OR 3.34 (95% CI: 1.97, 5.66); I^2 0%] subpopulations, the effect estimate was significant, but not for North-East Asians [OR 8.00 (95% CI: 0.49, 131.37)].

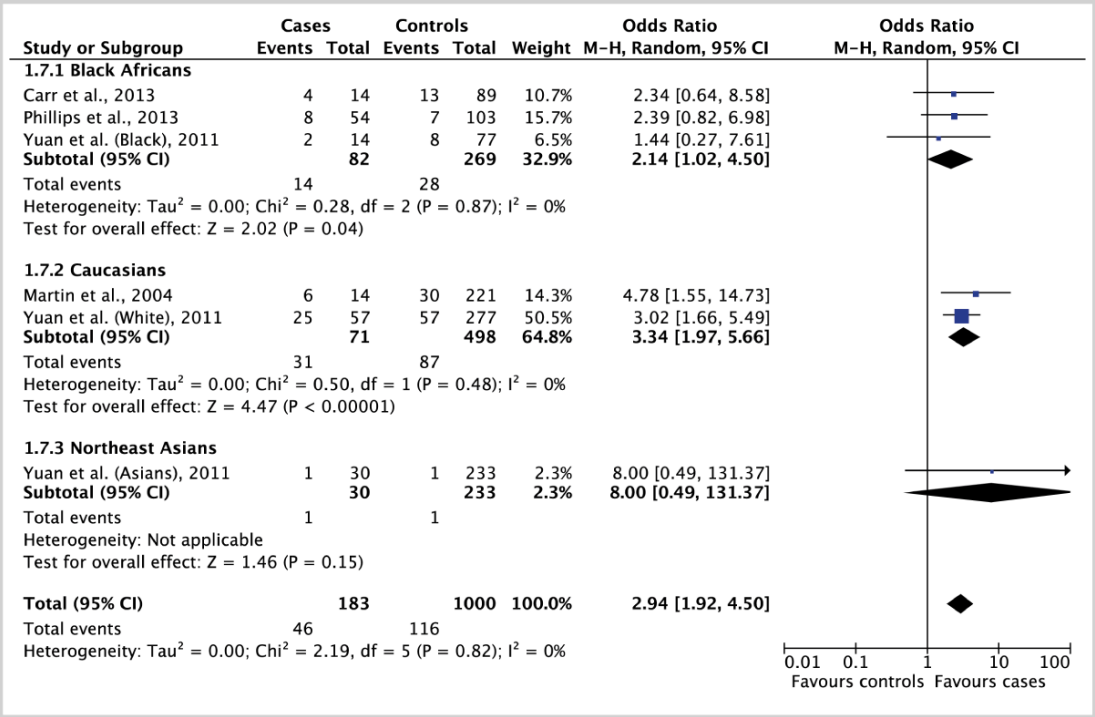


Fig. 6.5: Analysis of NVP-induced hepatotoxicity and *HLA-DRB1*01* in different populations

Data analysis was performed using the Mantel-Haenszel method. Events describe the number of carriers. The size of squares is proportional to the sample size. 95% CI is indicated by horizontal lines. Diamonds represent pooled ORs and heterogeneity is measured I^2. *CI* confidence interval, *HLA* human leukocyte antigen

Four studies investigated the associations of *HLA-DRB1*01* and cutaneous, hepatic and systemic HSRs in Caucasians [presented in figure 6.6 (GOZALO *et al.*, 2011; MARTIN *et al.*, 2005; VITEZICA *et al.*, 2008; YUAN *et al.*, 2011)]. The pooled effect estimate was significant [OR 3.70 (95% CI: 1.46, 9.39); I^2 52%], although there was substantial heterogeneity between studies.

The analysis of *HLA-DRB1*01:02* and hepatotoxicity in black African hypersensitive patients and tolerant controls was investigated in two studies [as shown in figure 6.7 (CARR *et al.*, 2013; PHILLIPS *et al.*, 2013)]. However, the pooled effect was not significant [OR 2.42 (95% CI: 0.59, 9.92); I^2 48%] and there was moderate heterogeneity between studies.

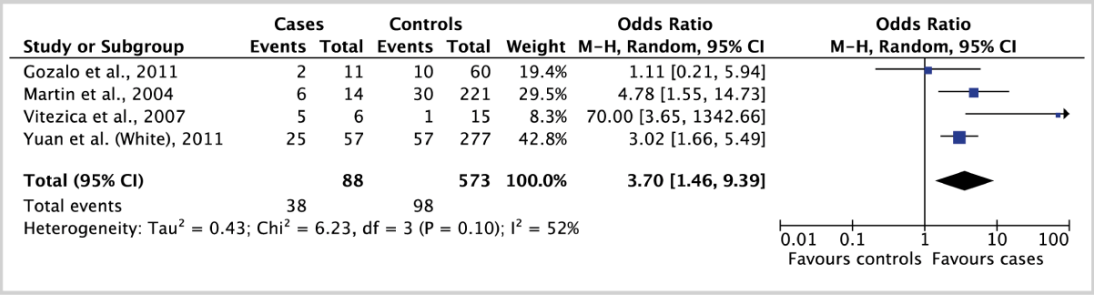


Fig. 6.6: Forest plot for association between NVP-induced hypersensitivity and *HLA-DRB1*01* in Caucasians

Data was analysed using the Mantel-Haenszel method. Carriers are reported as events. The 95% CI is represented by the horizontal line and the size of the square corresponds to the sample. The pooled estimate is represented by a diamond shape. Heterogeneity is measured by I^2. *CI* confidence interval, *HLA* human leukocyte antigen

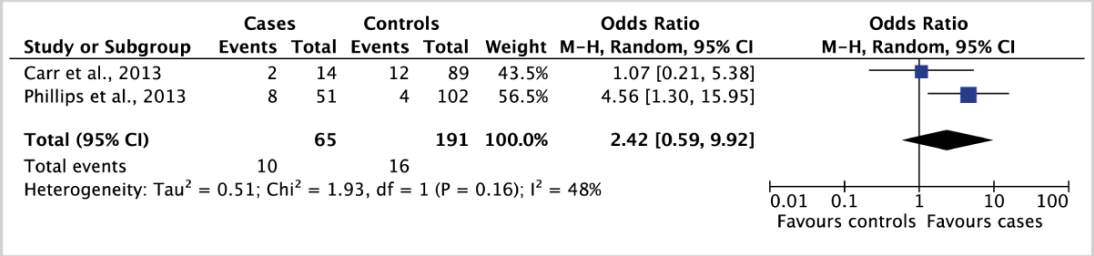


Fig. 6.7: Association between NVP-induced hepatotoxicity and *HLA-DRB1*01:02* in Black Africans

Forest plot for the analysis of HLA-DRB1:02 and hepatotoxicity using the Mantel-Haenszel method. Events represent the number of carriers. The 95% CI is represented by the horizontal line and the size of the squares correlates to the sample size. A diamond shape represents the pooled effect estimate. Heterogeneity is measured by I^2. *CI* confidence interval, *HLA* human leukocyte antigen

Results not included in meta-analysis

Two studies reported outcomes only as HLA-allele frequency (LITTERA *et al.*, 2006; UMAPATHY *et al.*, 2011) and although these data support the association between specific HLA genotypes and NVP hypersensitivity and are as such included in this systematic review, the results could not be pooled in a meta-analysis with any data provided by other reports. At the time of this review no additional data were made available after contacting the corresponding authors of these studies. In addition, the study by Gatanaga *et al*. could not be included in the meta-analysis as it is the only study to have reported an association with

*HLA-C*08* (GATANAGA *et al.*, 2007). Of these three studies, two reported a significant association of *HLA-C*08* with NVP hypersensitivity in Caucasians [p = 0.004 (LITTERA *et al.*, 2006)] and North-East Asians [p = 0.03 (GATANAGA *et al.*, 2007)]. The association between *HLA-B* alleles and NVP-induced HSRs were reported for *HLA-B*35* and *HLA-B*14* in South Asians [p = 0.003 (UMAPATHY *et al.*, 2011)] and Caucasians [p = 0.004 (LITTERA *et al.*, 2006)] respectively.

One study reported a significant association between the *HLA-B*35-C*04* haplotype and cADRs in North-East and South-East Asians [p < 0.001 (YUAN *et al.*, 2011)], whereas a different study conducted in Caucasians showed a significant association with *HLA-B*14-C*08* and NVP-induced hypersensitivity [p = 0.004 (LITTERA *et al.*, 2006)]. Due to insufficient data, these results could not be included in the meta-analysis.

6.4 Discussion

Adverse drug reactions represent a major complication in the provision of HAART. In general, ADRs account for 6.5% of hospital admissions and represent a significant cause of patient morbidity while they have also been associated with lower adherence to HIV treatment (ICKOVICS and MEISLER, 1997; LAZAROU *et al.*, 1998; PIRMOHAMED *et al.*, 2002). The identification of *HLA-B*57:01* as a high-risk allele for ABC hypersensitivity led to the implementation of *B*57:01* screening prior to ABC therapy and has thus significantly reduced the incidence of HSRs to ABC (ZUCMAN *et al.*, 2007). Several HLA-alleles have been associated with HSRs to NVP but none of these associations have the predictive accuracy or specificity seen with the association between *HLA-B*57:01* and ABC hypersensitivity. Between study variability in ethnic backgrounds, alleles investigated and definition and diagnosis of HSRs may contribute to the differences seen between studies in terms of alleles significantly associated with NVP hypersensitivity. To assess the overall effect of specific HLA-alleles on the risk of NVP-induced HSRs, a systematic review and meta-analysis were undertaken which provided the means to systematically identify and quantify genetic associations across different subgroups of patients.

Risks of bias and study limitations

Twelve studies investigating the association between HLA genotype and NVP hypersensitivity have been identified. All studies included in this review were significantly smaller than the calculated sample size requirements by Jorgensen *et al.* (2008), which is likely due to the rarity of NVP-induced hypersensitivity reactions, but still provided enough statistical power to show positive associations with certain HLA-alleles. Still some of the studies may be underpowered to provide sufficient information on additional variants or associated genotypes (JORGENSEN and WILLIAMSON, 2008).

The variety of HLA genotyping techniques used (such as serological and sequence based HLA typing), genotype resolution and partial reporting of genotypes may all contribute to the differences in associations described. Low resolution typing refers to HLA-alleles reported at a two-digit level (e.g.: *C*04*) that represents a wide group of alleles. In addition, ambiguous results generated by high resolution HLA typing may not lead to the identification of two specific alleles but rather in a list of possibilities, e.g. *HLA-C*04:01/04:05/04:07*. The majority of studies had to be grouped at two-digit level to allow a comprehensive analysis of sub-group specificities. But this does not support the identification of individual alleles associated with NVP-induced ADRs. In addition, bias due to the selective reporting of statistically significant risk alleles and investigated genotypes cannot be excluded. Six of the twelve studies included in this review analysed both MHC class I and class II alleles (CARR *et al.*, 2013; CHANTARANGSU *et al.*, 2009; GAO *et al.*, 2012; GATANAGA *et al.*, 2007; LITTERA *et al.*, 2006; YUAN *et al.*, 2011). However data were often only described for the statistically significant HLA associations (CHANTARANGSU *et al.*, 2009; GATANAGA *et al.*, 2007; LITTERA *et al.*, 2006; YUAN *et al.*, 2011). The remaining six studies investigated specific HLA-alleles based on previously published results (GOZALO *et al.*, 2011; LIKANONSAKUL *et al.*, 2009; MARTIN *et al.*, 2005; PHILLIPS *et al.*, 2013; UMAPATHY *et al.*, 2011; VITEZICA *et al.*, 2008), introducing a selection bias of the alleles represented in these studies. Thus, the specificity of the associations described in this study is largely restricted by the nature of the published data. Furthermore, HLA-allele frequencies and LD patterns have been shown to vary

amongst different populations (CAO *et al.*, 2001; DE BAKKER *et al.*, 2005) and therefore the observed associations may be affected by the population-specific frequency of the HLA-alleles, which requires a clear classification of patient groups.

Heterogeneity may also be introduced to the analysis by the large variety of adverse events associated with NVP, which demands a precise diagnosis and definition of phenotypes. Recently, standardised sets of phenotypic requirements have been proposed for severe immune-mediated cADRs [the Phenotype Standardization Project (PIRMOHAMED *et al.*, 2011)] and DILI (AITHAL *et al.*, 2011). Phenotype standardisation allows a precise identification of drug-induced adverse events promoting "adequate and accurate patient recruitment" into pharmacogenetic, immunological and epidemiological studies, and thus should be considered and encouraged in all studies (PIRMOHAMED *et al.*, 2011). Due to diversity of definitions of NVP-induced adverse events in the literature, the organisation of phenotypes had to be simplified in order to analyse the effect of certain HLA-alleles across different populations. Adverse reactions were classified primarily by the occurrence of cutaneous or hepatic events regardless of the manifestation of systemic symptoms [e.g.: hepatotoxicity with or without associated reactions (PHILLIPS *et al.*, 2013) was grouped together with DILI (CARR *et al.*, 2013); see table 6.2 or appendix 2 for further classifications]. This represents a limitation of our study, as no sub-phenotypes could be classified and individually assessed, although the study by Carr *et al.* (2003) indicated that one particular allele may be significant for one specific reaction. It was shown that *HLA-C*04:01* positive patients showed a higher probability to develop NVP-induced SJS/TEN [OR 17.52 (95% CI: 3.31 – 92.8)] than general hypersensitivity [OR 2.64 (95% CI: 1.13 – 6.18); (CARR *et al.*, 2013)].

Meta-analyses

Our study indicates that all reported HLA-alleles, apart from *HLA-DRB1*01:02*, are significantly associated with an increased risk of developing HSRs to NVP (summarised in table 6.3). The risk of cADRs is higher in *HLA-B*35* and *HLA-*

*C*04* positive patients compared to those without these alleles [OR 2.45 (95% CI: 1.10, 5.48) and OR 2.63 (95% CI: 1.97, 3.52) respectively]. Hepatotoxicity is more likely to occur in *HLA-B*58:01* black African [OR 3.51 (95% CI: 1.72, 7.19)] or *HLA-DRB1*01* positive patients of Asian, black African or Caucasian ancestry [OR 2.94 (95% CI: 1.92, 4.50)]. Additionally, *HLA-DRB1*01* is also associated with an increased risk of HSRs in Caucasians [OR 3.70 (95% CI: 1.46, 9.39)]. Due to the overlap of studies included in the two analyses of *HLA-DRB1*01*, it cannot be excluded that this association is predominantly driven by patients experiencing NVP-induced hepatotoxicity. The association between *HLA-B*35* is further supported by a study conducted in Indian patients that shows *B*35* to be significantly associated with NVP-induced HSRs (UMAPATHY *et al.*, 2011). Further reports have shown a statistically significant association between *HLA-B*14* [Caucasians (LITTERA *et al.*, 2006)] and *HLA-C*08* with NVP hypersensitivity [Caucasians (LITTERA *et al.*, 2006) and North-East Asians (GATANAGA *et al.*, 2007)]. Although these studies could not be included in the meta-analysis they do evidently support the association of several MHC class I and class II alleles and NVP-induced adverse events.

Due to the strong LD between the *HLA-B*35*, *C*04:01* and *DRB1*01:01* alleles, this haplotype has been defined as an 'extended' or 'ancestral' haplotype which has been shown to be preserved from early Caucasian populations (ABRAHAM *et al.*, 1992; SANCHEZ-MAZAS *et al.*, 2000). Thus, further studies are needed to define whether the associations of *HLA-B*35*, *C*04:01* and *DRB1*01:01* and NVP-induced hypersensitivity are allele or haplotype dependent effects.

Table 6.3: Summary of overall pooled effect estimates

HLA-allele	# of studies included	Type of HSR	Population	OR	95% CI	I^2
*HLA-B*35*	3	cADRs	Mixed	**2.45**	[1.10, 5.48]	**69%**
*HLA-B*58:01*	2	Hepatotoxicity	Black Africans	**3.51**	[1.72, 7.19]	0%
*HLA-C*04*	3	cADRs	Mixed	**2.63**	[1.97, 3.52]	0%
	4	All phenotypes	Mixed	**2.59**	[1.95, 3.43]	0%
*HLA-DRB1*01*	4	All phenotypes	Caucasians	**3.70**	[1.46, 9.39]	**52%**
	4	Hepatotoxicity	Mixed	**2.94**	[1.92, 4.50]	0%
*HLA-DRB1*01:02*	2	Hepatotoxicity	Black Africans	2.42	[0.59, 9.92]	**48%**

Significant associations are reported in bold. *CI* confidence interval, *HLA* human leukocyte antigen, *OR* odds ratio, I^2 measurement of heterogeneity

Considerable heterogeneity was reported for the association of *HLA-B*35* (I^2: 69%), *-DRB1*01* (I^2: 52%) and *-DRB1*01:02* (I^2: 48%) and NVP hypersensitivity. Although two of the three studies showed a significant association between the HLA-allele and the NVP-induced HSR investigated (see table 6.3), these associations should be interpreted cautiously. The heterogeneity observed in the meta-analysis of *HLA-B*35* and cADRs may be explained by the small number of carriers included in the study by Yuan *et al.* (2011). In contrast, the in between study heterogeneity observed in the analysis of *HLA-DRB1*01* and *HLA-DRB1*01:02* in NVP-treated patients of Caucasian and black African ancestry may be based upon publication bias; both the study by Gozalo *et al*. (2011) and Carr *et al*., (2013) report a non-significant association of these alleles and NVP-induced HSRs. Moreover, no individual data was available for the *HLA-DRB1*01:01* negative patients included in the study by Gozalo *et al*. (2011) and Phillips *et al.* (2013) making it difficult to identify the specific number of *DRB1*01* carriers.

The non-significant associations observed in some of the subgroup analyses, particularly in North-East Asians (for *HLA-B*35*, *-C*04* and *-DRB1*01*) as well as black Africans (see *HLA-B*35* and *-DRB1*01*) and Caucasians (*HLA-B*35*) are due to the small number of carriers reported for these associations, which is further supported by the wide CIs. The pooled effect estimates reported in association with NVP hypersensitivity are significantly lower than those reported in association with *HLA-B*58:01* and allopurinol [OR 96.60 (95% CI: 24.49, 381.00); (SOMKRUA *et al.*, 2011)] or *HLA-B*15:02* and CBZ-induced SJS/TEN [OR 113.39 (95% CI: 51.24, 250.97); (YIP *et al.*, 2012)]. Although these studies may not investigate the same associations and drugs, they illustrate clearly that more detailed data are needed to evaluate the use of pre-prescription genotyping. This was successfully applied for ABC and CBZ treatment (CHEN *et al.*, 2011; MALLAL *et al.*, 2008).

No HLA-allele frequency was available for the different studies included in this report. The comparison of allele frequencies of Asians, black Africans and Caucasians (as summarised in table 6.4) acquired from public databases did not

provide enough data to specify whether the differing allele frequencies affect certain associations.

Table 6.4: Overview of allele frequencies

Population	*HLA-B*35*	*HLA-B*58:01*	*HLA-C*04*	*HLA-DRB1*01*	*HLA-DRB1*01:02*
Black Africans	0.03 - 0.18	0.02 - 0.10	0.11 - 0.21	0.07 - 0.10	0.02 - 0.08
Caucasians	0.06 - 0.16	0.01 - 0.06	0.09 - 0.18	0.07 - 0.15	0.01 - 0.04
North-East Asians	0.02 - 0.07	0.02 - 0.07	0.01 - 0.02	0.01 - 0.02	NA
Thai	0.05	0.08	0.01	0.01 - 0.12	NA

Data acquired from www.allelefrequencies.net (GONZALEZ-GALARZA *et al.*, 2011); *HLA* human leukocyte antigen, *NA* not available

It is worth noting that the method used in this study to assess in between study heterogeneity is rather simplified. Together with the relatively limited number studies, quantity of different HLA-alleles, genotyping techniques and resolutions as well as the various phenotype definitions used and subpopulations described, the causes of heterogeneity cannot be addressed specifically. A profound study of associations and sources of heterogeneity could be accomplished by an individual patient data (IPD) meta-analysis (will be discussed in chapter 7 in more detail),

Chapter 7

Final discussion

Drug-induced adverse reactions represent a major complication in the administration of medical therapy. In general, ADRs account for 6.5% of hospital admissions in the UK (PIRMOHAMED *et al.*, 2004), whereas in the US drug-related adverse effects are a leading cause of patient morbidity (LAZAROU *et al.*, 1998). Idiosyncratic drug reactions are unpredictable and sometimes life threatening reactions, which can affect single organs as well as multiple organ systems (UETRECHT and NAISBITT, 2013). These reactions represent a common cause of drug withdrawal and have been associated with reduced adherence to HAART (MAX and SHERER, 2000). However, the complex interactions between drugs, genomic variations, individual differences in the immune response and other physiological and environmental factors have not been clearly established. Recent studies suggest that combinations of NVP-reactive MHC class I restricted CD8+ T cells and class II restricted CD4+ T cells may, amongst other factors, contribute to the development of NVP-induced HSRs (see chapter 1.5.3). However, the inconsistencies among the genetic and immunological associations indicate that population-specific differences and the phenotypic diversity of HSRs may influence the characteristics of these reactions.

In all populations, regardless of ethnicity, the development of adverse effects affecting the skin and liver with NVP is considered to be immune mediated. Particularly the association of higher CD4+ T cell counts at treatment initiation with an increased risk of HSRs (BOEHRINGER-INGELHEIM, 2012; CARR *et al.*, 2013; TAIWO, 2006) as well as the histopathological findings of immune cell infiltration in lesional areas from patients further support this hypothesis (PICHLER, 2003; WETTER and CAMILLERI, 2010).

To gain more insight into the molecular mechanisms underlying the immune reaction and inflammatory response of NVP-induced hypersensitivity, mRNA expression profiles of NVP-treated patients from Malawi were analysed (chapter 2). Similar to the results by Bellón *et al.* (2010), we found the expression of DAMPs significantly upregulated in NVP-treated patients (BELLON *et al.*, 2010). These molecules comprise a diverse group of genes that have been shown to be upregulated in various inflammatory conditions [e.g. Crohn's

disease (LOTZE and TRACEY, 2005) and arthritis (FOELL *et al.*, 2007)] and are known to stimulate pattern recognition receptors. Thus, early innate and adaptive immune responses are triggered. These reports support the concept of similar immunological and inflammatory mechanisms taking place in patients, and specifically in patients with drug-induced hypersensitivity, regardless of the causative agent (e.g. drug or initial tissue damage).

Among the molecules with the highest increase in expression was *CD177*, a neutrophil specific antigen shown to promote transendothelial migration of CD177-positive neutrophils through heterophilic interaction with PECAM-1 (BAYAT *et al.*, 2010; SACHS *et al.*, 2007). Studies have shown that the expression, as well as the proportion of CD177-positive granulocytes, is associated with a number of *CD177* polymorphisms (MORITZ *et al.*, 2010; WOLFF *et al.*, 2003). These results could only be partially replicated for rs10425835 in our heterogeneous cohort of healthy volunteers (chapter 3). However, as the necessary reagents were not commercially available, the effects of the rs61625631 polymorphism as well as 7D8 antibody clone could not be investigated. To fully characterise the association of *CD177* polymorphisms and its surface expression, these results would need to be replicated in a larger study of equally sized cohorts of Caucasian and black African patients. This may allow possible ethnic differences in allele frequency and baseline protein expression to be uncovered.

CD177 mRNA expression has been shown to be strongly upregulated during infection, inflammation and several blood related disorders (BUX *et al.*, 1996; GOHRING *et al.*, 2004; LILL *et al.*, 2013; MONTASER *et al.*, 2011; SHAHABI *et al.*, 2013; SIRHAN *et al.*, 2005). Similar to these results and the observation by Bellón *et al.* (2010) in generalised severe cutaneous reactions, gene expression of *CD177* was strongly upregulated in NVP-treated patients from Malawi as well as after *in vitro* treatment of patient samples collected in Liverpool (chapter 2). In contrast, no NVP-dependent increase in CD177 surface expression was found following *in vitro* treatment of NVP-hypersensitive patient samples, which is consistent with previous findings (SLEZAK *et al.*, 2009; TEMERINAC *et al.*, 2000). Also, a case-control study of HIV positive, NVP-hypersensitive and tolerant

patients did not reveal an association between CD177 genotype and NVP hypersensitivity (chapter 3).

Our results suggest that the NVP-dependent increase in CD177 gene expression is unlikely to be associated with the preferential transmigration of CD177-positive neutrophils. Although further studies investigating the effect of NVP treatment on the transendothelial migration of neutrophils are needed, CD177 may intensify the inflammatory response similar to other DAMPs, which have been shown to be preformed and released either directly by immune cells or following necrosis. These endogenous molecules proceed to recruit and stimulate cells of the innate immune systems, and thus the formation of proinflammatory cytokines through the activation of the NF-κB or MAPK pathways. As a result, DAMPs indirectly activate cells of the adaptive immune system, promoting and accelerating the inflammatory response (BIANCHI, 2007; ZHENG *et al.*, 2011). Extensive work is needed to elucidate which pathways lead to the common overexpression of endogenous molecules, such as DAMPs, in drug hypersensitive patients. Furthermore the role of these molecules in the pathogenesis of drug-induced adverse reactions needs to be investigated. The interaction of the innate and adaptive immune system in the development of HSRs may provide the necessary clues to unravel the complex mechanisms that define the individual susceptibility of patients.

The associations between specific HLA allelotypes and drug hypersensitivity reactions represents one of the greatest advancements in the field of pharmacogenomics, adding evidence to the immunopathogenic mechanisms involved in drug-induced HSRs (see 1.3.3). Numerous MHC class I and class II alleles have been described as risk factors for NVP hypersensitivity (chapter 1.5.3). However, none of these associations offer the predictive accuracy and independent replication observed with the associations of *HLA-B*57:01* and HSRs to ABC (MALLAL *et al.*, 2008) or *HLA-B*15:02* and CBZ-induced SJS/TEN (CHEN *et al.*, 2011). A potential explanation for this may be that for some but not all drugs, associations may not be generalised across different ethnicities and

may be specific for one type of HSRs alone. This makes it more difficult to implements genetic testing into clinical practice (PHILLIPS and MALLAL, 2010).

Our meta-analysis of studies investigating the role of HLA-alleles in NVP-induced adverse events identified a total of four alleles (*HLA-B*35*, *-B*58:01*, *-C*04*, and *-DRB1*01*) as risk associated factors for different types of HSRs (chapter 6). The analysis indicates that *HLA-C*04* carriage may be a common susceptibility factor for NVP-associated cADRs across multiple ethnicities, whereas the reported risk alleles *HLA-B*35, HLA-B*58:01* and *HLA-DRB1*01* appear to be population-specific (in Thai, black African and Caucasian respectively). The considerable heterogeneity observed in a number of analyses may be explained by the inconsistent phenotype definitions used in the studies. This highlights the need for a standardised definition of outcomes and subpopulations in order to decrease clinical and methodological diversity between studies. To facilitate cross-study comparisons, phenotypes of cADRs and DILI should be standardised as per previous published guidelines (AITHAL *et al.*, 2011; PIRMOHAMED *et al.*, 2011). Additionally, the methodological quality of genetic association studies should be addressed and adhered to (JORGENSEN and WILLIAMSON, 2008).

The application of an IPD meta-analysis could improve the accuracy and quality of the reported associations between HLA-alleles and NVP hypersensitivity, and thus produce more reliable results. It would allow the verification of the results presented in the original study, while at the same time may lead to the identification of new associations. In addition, phenotype definitions and the statistical analysis can be standardised across all studies. Also, missing data can be accounted for in greater detail than in a conventional meta-analysis of pooled data. In order to facilitate this next step, a search strategy was therefore expanded to include clinical trials and reports in which only allele frequency data was presented. A total of 13 studies (listed in table 7.1) conducted by 12 research groups have been identified and the corresponding first or last author of each study contacted via email. Raw data will be collected and the standardisation of outcomes and genotypes will allow us to investigate the effect of common alleles across different subgroups. In addition to common

alleles, haplotypes will be investigated to further identify common risk associations between populations and thus maybe provide a connection between the different HLA associations observed. Further, the effect of population-specific allele frequencies on associated HLA-alleles will be investigated as part of the IPD meta-analysis.

Table 7.1: Studies and trials identified for individual patient data meta-analysis

Title	Reference
Predisposition to nevirapine hypersensitivity associated with HLA-DRB1*0101 and abrogated by low CD4 T-cell counts.	(MARTIN *et al.*, 2005)
HLA-dependent hypersensitivity to nevirapine in Sardinian HIV patients.	(LITTERA *et al.*, 2006)
HLA-Cw8 primarily associated with hypersensitivity to nevirapine.	(GATANAGA *et al.*, 2007)
HLA-DRB1*01 associated with cutaneous hypersensitivity induced by nevirapine and efavirenz.	(VITEZICA *et al.*, 2008)
HLA-B*3505 allele is a strong predictor for nevirapine-induced skin adverse drug reactions in HIV-infected Thai patients.	(CHANTARANGSU *et al.*, 2009)
HLA-Cw*04 allele associated with nevirapine-induced rash in HIV-infected Thai patients.	(LIKANONSAKUL *et al.*, 2009)
HLA-Dependent Hypersensitivity Reaction to Nevirapine in Chinese Han HIV-Infected Patients.	(GAO *et al.*, 2012)
Pharmacogenetics of toxicity, plasma trough concentration and treatment outcome with nevirapine-containing regimen in anti-retroviral-naive HIV-infected adults: an exploratory study of the TRIANON ANRS 081 trial.	(GOZALO *et al.*, 2011)
HLA involvement in nevirapine-induced dermatological reaction in antiretroviral-treated HIV-1 patients.	(UMAPATHY *et al.*, 2011)
Toxicogenomics of nevirapine-associated cutaneous and hepatic adverse events among populations of African, Asian, and European descent.	(YUAN *et al.*, 2011)
Association of Human Leukocyte Antigen Alleles and Nevirapine Hypersensitivity in a Malawian HIV-Infected Population.	(CARR *et al.*, 2013)
Associations between HLA-DRB1*0102, HLA-B*5801, and hepatotoxicity during initiation of nevirapine-containing regimens in South Africa.	(PHILLIPS *et al.*, 2013)
Genotype Based Personalized Prescription of Nevirapine (GENPART); NCT00986063	(MAHASIRIMONGKOL, 2013)

Although the genetic associations between NVP hypersensitivity and HLA-alleles have been extensively investigated, nothing is known about possible effects of HLA expression levels on the development of NVP-induced ADRs. In our cohort of NVP-treated patients from Malawi, studies have shown that carriers of *HLA-C*04:01* are at higher risk of NVP-induced HSRs, and particularly

cutaneous adverse events (CARR *et al.*, 2013). These results are consistent with the meta-analysis results reported above and have been further supported by a GWAS that identified a SNP within the *HLA-C* locus to be significantly associated with NVP-induced SJS/TEN (manuscript in preparation). Variations in HLA-C expression have been shown to affect the HIV host response and these variations can be partially explained by the post-transcriptional regulation of *HLA-C* by miR-148a (INTERNATIONAL HIV CONTROLLERS STUDY *et al.*, 2010; KULKARNI *et al.*, 2011; THOMAS *et al.*, 2009). Contradictory to our expectations, expression analysis of circulatory miR-148a levels showed an increase in miR-148a expression at time of reaction (chapter 4). The functional implications of these results will have to be further analysed (see 4.4). However, it has not been confirmed if the expression levels of miR-148a in serum correspond to miR-148a expression in PBMCs of NVP-hypersensitive patients. Ideally, miR-148a expression would be analysed in freshly isolated PBMCs of acute NVP-hypersensitive patients and tolerant controls. Differences in miR-148a expression levels could then be correlated to *HLA-C* gene and protein expression. As no specific HLA-C*04:01 antibody is commercially available, HLA-typing is needed to distinguish between individuals carrying "inhibited" *HLA-C* alleles and "escape" alleles.

Functional studies investigating the pharmacogenetic association of *HLA-B*57:01* and ABC hypersensitivity have shown that B*57:01 restricted CD8+ T cells can be isolated from patients with a history of ABC-induced HSRs (CHESSMAN *et al.*, 2008). Given these results, the immunogenic capacity of NVP and its metabolites could be investigated in isolated T cells from *HLA-C*04:01* positive, NVP-hypersensitive patients. Using a *HLA-C*04:01* transfected, MHC class I-deficient B lymphoblastoid cell line (CR1) as APCs, T cells could be stimulated in the presence or absence of the drug *in vitro*. T cell activation would then be investigated using a proliferation assay, which measures the proliferative response of sensitised T cells by 3H-thymedine uptake (NYFELER and PICHLER, 1997). To further define NVP-specific T cells, an enzyme-linked immunospot (ELISpot) assay could be used to quantitatively analyse cytokine secretion. Likewise, intracellular cytokine staining (ICS) would allow the quantification and differentiation of cytokine-producing CD4+ and CD8+ T cells

using flow cytometry (NOMURA *et al.*, 2008). Taken together, these methods may help to detect the immunogenic potential of NVP and to functionally characterise NVP-specific T cells as well as the role of specific HLA-alleles associated with HSRs to NVP.

MicroRNA-dependent gene regulation has been shown to be of considerable importance for a wide range of biological functions, such as cell proliferation, immune response, oncogenesis and cell death (FABBRI, 2013; JOHNSON *et al.*, 2007; TOMANKOVA *et al.*, 2011; XU *et al.*, 2004). Using a serum-specific PCR array, the expression profiles of 84 miRNAs were investigated and a total of 21 miRNAs were found to be differentially expressed in NVP-hypersensitive and tolerant patients following treatment initiation (see chapter 5). Expression levels of miR-205 were significantly increased in acute, NVP-hypersensitive samples. Whether miR-205 plays a part in the pathogenesis of NVP-induced cADRs or serum miR-205 levels could be used as a diagnostic marker for these reactions remains unclear (see 5.4).

Using a systems biology approach, miRNA expression profiles could be combined with predicted and experimentally proven interactions to construct a regulatory network (WATANABE and KANAI, 2011). Data would be retrieved from relevant publications and databases and organised in a regulatory map. Computational miRNA target prediction databases [e.g. www.targetscan.org or miRanda software(ENRIGHT *et al.*, 2003)] could be used to detect a large number of miRNA-mRNA interactions. Additionally *in vitro* studies targeted at the identification of mRNAs, which are either overexpressed or inhibited by the application of miRNA inhibitors and mimics, would allow the identification of direct miRNA target molecules. Based on the regulatory network a mathematical model could then be constructed, allowing the investigation of specific features of the biological pathways embedded within the network. Combined, these methods may determine common miRNA-mRNA interactions and thus expose miRNAs functions in regulatory networks that define the pathogenic pathways underlying NVP hypersensitivity (KHATRI *et al.*, 2012).

In recent years, research into drug-induced hypersensitivity has progressed extensively. The discovery of strong associations between *HLA-B*57:01* and ABC-induced HSRs or *B*15:02* and CBZ-induced SJS/TEN led to the clinical implementation of pharmacogenetic screening as a standard of care approach and have been shown to reduced the incidence of drug-induced HSRs in certain populations (CHEN *et al.*, 2011; MALLAL *et al.*, 2008). In addition, *in vitro* studies have shown that the interactions between ABC and MHC molecule lead to a modulated T cell response (ILLING *et al.*, 2012; NORCROSS *et al.*, 2012; OSTROV *et al.*, 2012). However, it is unlikely that these results can be generalised for different drugs. A better understanding of the molecular mechanisms involved in the pathophysiology of drug-induced adverse reactions may improve the characterisation of true causative associations and pathways.

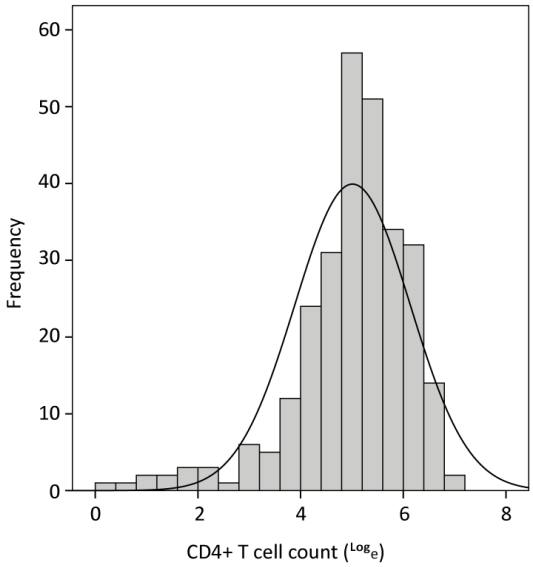
Nevirapine is generally used as a first-line regimen in resource-limited settings. Since the initiation of the "provision of ART and good management of HIV-related diseases to HIV-infected patients" program in 2006, the number of patients initiated on NVP-containing ART has steadily increased in Malawi (DEPARTMENT FOR HIV AND AIDS, 2012). Yet, variations among the genetic associations observed in NVP hypersensitivity across different populations emphasise the need for further research to define common pathways of ADRs. This thesis demonstrates that the contributing factors of NVP-induced adverse reactions are extremely complex. Whether such complexity can be fully incorporated into one predictive clinical model and utilised for different populations remains to be seen. Given the limited healthcare resources in Malawi, a detailed economic evaluation would be needed to assess if a predictive test could be implemented as a standard of care approach. Further work in this area may however provide valuable insight in the immunological and cellular pathways involved in drug-induced HSRs and ultimately improve the efficacy and safety of clinical therapy.

Appendices

Contents

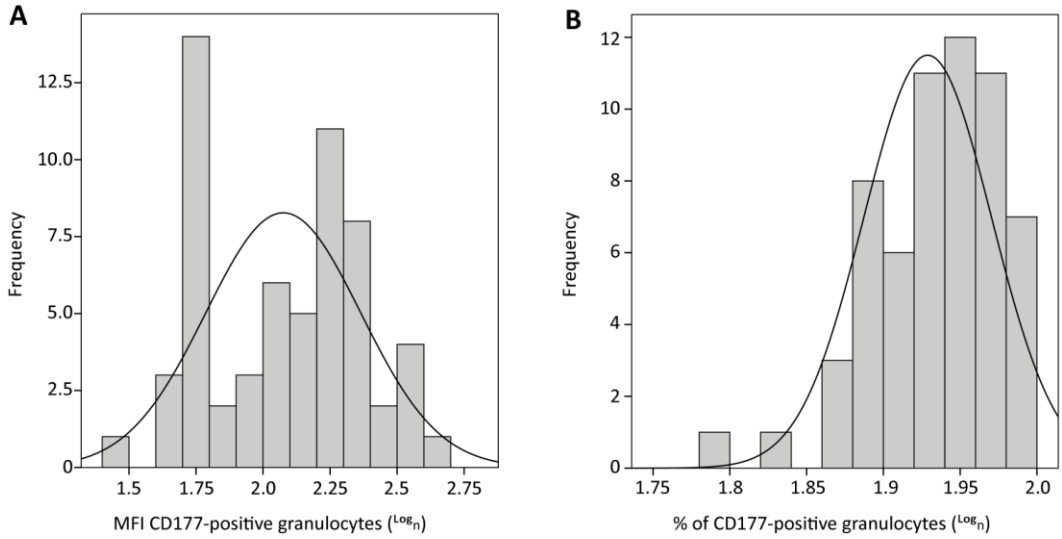
Appendix 2.1: *Normal distributed CD4+ T cell count*

Data was normally distributed after $\log_e$ transformation.



Appendix 2.2: *Histogram showing normally distributed MFI and % of CD177-positive granulocytes*

Data was normally distributed after $\log_{10}$ transformation. Figure **A** shows the distribution of the MFI and **B** of the proportion of CD177-positive granulocytes.



Appendix 3.1: *CD177 PCR and extension primers for SNP genotyping using Sequenom MassARRAY iPLEX platform*

SNP	Forward primer (5' → 3')	Reverse primer (5' → 3')	Amplicon length	Extension primer sequence (5' → 3')
rs10425835	ACGTTGGATGAGGTGTGTCAGGAGACGCT	ACGTTGGATGGACAGGTGTTCGTCTTCTAC	90	TCCACGTACCTACATCTA
rs12462403	ACGTTGGATGATGTCATTGAAAGCAATGG	ACGTTGGATGTCATAGTCTGAGTCAGACAC	100	TGGAAGACTTTACAAAAAAAAAA
rs45441892	ACGTTGGATGAGAGATTACCAGCCACAGAC	ACGTTGGATGTGGCAGTGGGAGGATGAAC	96	GGCCAGCAGTAATACCG
rs45553433	ACGTTGGATGGTGTTCTTAGGGGTCCATTG	ACGTTGGATGTGCTCTGCCAGTTTGGGAC	96	ggacGTTTGGGACAGTTCAGC
rs4803613	ACGTTGGATGTTTCCAGCCTCTCAGCCTAC	ACGTTGGATGAGGCAAGCGAGGATGGAGG	100	GCGAGGATGGAGGTCAGGC
rs6509088	ACGTTGGATGCAATGTTCTTGCTTGACGCC	ACGTTGGATGCAAAGCCAGTATGAGTTTAG	100	AGTTTAGTCAAGTCTGATTTCC
rs7257560	ACGTTGGATGGGAGAGGAGCCTAATGAGAA	ACGTTGGATGATCCAAGCTGGTTTGGAGAC	100	AATGAAGGGCAGGCTTT
rs73559882	ACGTTGGATGGTGTGTCATAATGGTGGTCC	ACGTTGGATGTTCCTGGCTTACACACACCC	99	GGGATTCCTCTCCCCAGAT
rs78718189	ACGTTGGATGTGGCACTGGCCCCAGCGC	ACGTTGGATGGGGTAATAGAGTTAGCAGG	81	GCAGGAAGGGCAAACCACTC

Appendix 5.1: *Plate layout of the miScript miRNA PCR Array Human Serum*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	let-7a	let-7a	miR-1	miR-1	miR-100	miR-100	miR-106	miR-106	miR-10b	miR-10b	miR-122	miR-122	miR-124	miR-124	miR-125b	miR-125b	miR-126	miR-126	miR-133a	miR-133a	miR-133b	miR-133b	miR-134	miR-134
B	let-7a	let-7a	miR-1	miR-1	miR-100	miR-100	miR-106	miR-106	miR-10b	miR-10b	miR-122	miR-122	miR-124	miR-124	miR-125b	miR-125b	miR-126	miR-126	miR-133a	miR-133a	miR-133b	miR-133b	miR-134	miR-134
C	miR-141	miR-141	miR-143	miR-143	miR-146a	miR-146a	miR-150	miR-150	miR-155	miR-155	miR-17-5p	miR-17-5p	miR-17-3p	miR-17-3p	miR-18	miR-18	miR-192	miR-192	miR-195	miR-195	miR-196a	miR-196a	miR-19a	miR-19a
D	miR-141	miR-141	miR-143	miR-143	miR-146a	miR-146a	miR-150	miR-150	miR-155	miR-155	miR-17-5p	miR-17-5p	miR-17-3p	miR-17-3p	miR-18	miR-18	miR-192	miR-192	miR-195	miR-195	miR-196a	miR-196a	miR-19a	miR-19a
E	miR-19b	miR-19b	miR-200a	miR-200a	miR-200b	miR-200b	miR-200c	miR-200c	miR-203	miR-203	miR-205	miR-205	miR-208	miR-208	miR-20a	miR-20a	miR-21	miR-21	miR-210	miR-210	miR-214	miR-214	miR-215	miR-215
F	miR-19b	miR-19b	miR-200a	miR-200a	miR-200b	miR-200b	miR-200c	miR-200c	miR-203	miR-203	miR-205	miR-205	miR-208	miR-208	miR-20a	miR-20a	miR-21	miR-21	miR-210	miR-210	miR-214	miR-214	miR-215	miR-215
G	miR-221	miR-221	miR-222	miR-222	miR-223	miR-223	miR-224	miR-224	miR-23a	miR-23a	miR-25	miR-25	miR-27a	miR-27a	miR-296	miR-296	miR-29a	miR-29a	miR-30d	miR-30d	miR-34a	miR-34a	miR-375	miR-375
H	miR-221	miR-221	miR-222	miR-222	miR-223	miR-223	miR-224	miR-224	miR-23a	miR-23a	miR-25	miR-25	miR-27a	miR-27a	miR-296	miR-296	miR-29a	miR-29a	miR-30d	miR-30d	miR-34a	miR-34a	miR-375	miR-375
I	miR-423	miR-423	miR-499a	miR-499a	miR-574	miR-574	miR-885	miR-885	miR-9	miR-9	miR-92a	miR-92a	miR-93	miR-93	let-7c	let-7c	miR-107	miR-107	miR-10a	miR-10a	miR-128	miR-128	miR-130b	miR-130b
J	miR-423	miR-423	miR-499a	miR-499a	miR-574	miR-574	miR-885	miR-885	miR-9	miR-9	miR-92a	miR-92a	miR-93	miR-93	let-7c	let-7c	miR-107	miR-107	miR-10a	miR-10a	miR-128	miR-128	miR-130b	miR-130b

Appendix 5.1: continued

K	miR-145	miR-145	miR-148a	miR-148a	miR-15a	miR-15a	miR-184	miR-184	miR-193a	miR-193a	miR-204	miR-204	miR-206	miR-206	miR-211	miR-211	miR-26b	miR-26b	miR-30e	miR-30e	miR-372	miR-372	miR-373	miR-373
L	miR-145	miR-145	miR-148a	miR-148a	miR-15a	miR-15a	miR-184	miR-184	miR-193a	miR-193a	miR-204	miR-204	miR-206	miR-206	miR-211	miR-211	miR-26b	miR-26b	miR-30e	miR-30e	miR-372	miR-372	miR-373	miR-373
M	miR-374a	miR-374a	miR-376c	miR-376c	miR-7	miR-7	miR-96	miR-96	miR-103a	miR-103a	miR-15b	miR-15b	miR-16	miR-16	miR-191	miR-191	miR-22	miR-22	miR-24	miR-24	miR-26a	miR-26a	miR-31	miR-31
N	miR-374a	miR-374a	miR-376c	miR-376c	miR-7	miR-7	miR-96	miR-96	miR-103a	miR-103a	miR-15b	miR-15b	miR-16	miR-16	miR-191	miR-191	miR-22	miR-22	miR-24	miR-24	miR-26a	miR-26a	miR-31	miR-31
O	cel-miR-39	cel-miR-39	cel-miR-39	cel-miR-39	SNO RD61	SNO RD61	SNO RD68	SNO RD68	SNO RD72	SNO RD72	SNO RD95	SNO RD95	SNO RD96A	SNO RD96A	RNU 6-2	RNU 6-2	miRTC	miRTC	miRTC	miRTC	PPC	PPC	PPC	PPC
P	cel-miR-39	cel-miR-39	cel-miR-39	cel-miR-39	SNO RD61	SNO RD61	SNO RD68	SNO RD68	SNO RD72	SNO RD72	SNO RD95	SNO RD95	SNO RD96A	SNO RD96A	RNU 6-2	RNU 6-2	miRTC	miRTC	miRTC	miRTC	PPC	PPC	PPC	PPC

The human serum array contains four replicates of each miRNA. Rows O and P contain the internal normalisation control cel-miR-39 as well as assays for different snoRNAs (O5 – O16 and P5 – P16). Wells O17 – O20 and P17 – P20 contain assays for the miRNA reverse transcriptase control (miRTC); wells O21 – O24 and P21 – 24 contain replicate positive PCR controls (PPC). Each colour represents one possible sample.

Appendix 6.1: *Comprehensive list of data extracted for final review and meta-analysis in chapter 6*

Study	Ethnicity or study location	New ethnicity	Phenotype	Phenotype definition	New phenotype	Reported HLA-alleles	# of cases		# of tolerant controls		# of population controls		Comments
							HLA pos	Total	HLA pos	Total	HLA pos	Total	
(Carr *et al.*, 2013)	Malawi	Black African	all HSRs	Maculopapular rash without systemic manifestations (NIR); rash with blistering eruptions and involvement of 2 mucous membranes (SJS/TEN); Widespread rash and systematic manifestations (HSS); Visible jaundice and abnormal ALT levels (DILI)	general hypersensitivity	*C*04*	51	102	40	154	NA	NA	
			NIR, HSS, SJS/TEN		cADRs	*C*04*	55	117	40	154	NA	NA	
			all HSRs		general hypersensitivity	*DRB1*01*	9	93	12	89	NA	NA	not significant association
			DILI		hepatotoxicity	*DRB1*01*	4	14	13	89	NA	NA	not significant association
			DILI		hepatotoxicity	*DRB1*01:02*	2	14	11	89	NA	NA	not significant association
			all HSRs		general hypersensitivity	*B*35*	6	117	7	154	NA	NA	not significant association
			NIR, HSS, SJS/TEN		cADRs	*B*35*	5	102	7	154	NA	NA	not significant association
			all HSRs		general hypersensitivity	*B*58:01*	16	117	17	154	NA	NA	not significant association
			DILI		hepatotoxicity	*B*58:01*	6	18	17	154	NA	NA	not significant association

Appendix 6.1: continued

Study	Ethnicity or study location	New ethnicity	Phenotype	Phenotype definition	New phenotype	Reported HLA-alleles	# of cases		# of tolerant controls		# of population controls		Comments
							HLA pos	Total	HLA pos	Total	HLA pos	Total	
(CARR *et al.*, 2013)	Malawi	Black African	all HSRs	Maculopapular rash without systemic manifestations (NIR); rash with blistering eruptions and involvement of 2 mucous membranes (SJS/TEN); Widespread rash and systematic manifestations (HSS); Visible jaundice and abnormal ALT levels (DILI	general hypersensitivity	*B*35-C*04* haplotype	5	102	7	154	NA	NA	not significant haplotype
			NIR, HSS, SJS/TEN		cADRs		6	18	7	154	NA	NA	not significant haplotype
(CHANTARANGSU *et al.*, 2009)	Thai	Thai	skin rash	skin rash within six months after treatment initiation	cADRs	*B*35:05*	25	143	2	181	1	142	all patients (first and second screening combined)
(GAO *et al.*, 2012)	Han Chinese	Northeast Asian	HSR	extensive skin rash, bullous skin lesions or skin manifestations combined with one or more of the following symptoms: fever or liver toxicity	general hypersensitivity	*C*04*	8	32	6	71	NA	NA	
						*C*08*	5	32	11	71	NA	NA	not significant association
						*DRB1*01*	0	32	4	71	NA	NA	not significant association

Appendix 6.1: continued

Study	Ethnicity or study location	New ethnicity	Phenotype	Phenotype definition	New phenotype	Reported HLA-alleles	# of cases		# of tolerant controls		# of population controls		Comments
							HLA pos	Total	HLA pos	Total	HLA pos	Total	
(Gozalo *et al.*, 2011)	Ethnicity not available but majority of trial participants were Caucasian	Caucasian	acute NVP toxicity	cutaneous and hepatic toxicity not always linked; either grade 3 or 4 cutaneous toxicity or ALT elevation greater three times ULN	general hypersensitivity	*DRB1*01:01*	2	11	10	60	NA	NA	negative association
(Likanonsakul *et al.*, 2009)	Thai	Thai	skin rash	apparent skin rash without liver toxicity	cADRs	*C*04*	13	39	9	60	NA	NA	
(Martin *et al.*, 2005)	Western Australia HIV Cohort (82 - 93% Caucasian)	Caucasian	Hepato-toxicity/ multi-system reactions	hepatotoxicity grade 2 or greater, ALT > 2.5 times ULN; multi-system reactions also involved rash or fever	hepatotoxicity	*DRB1*01:01*	6	14	30	221	NA	NA	
(Phillips *et al.*, 2013)	South Africa (120 black, 8 white, 19 mixed, 3 Asian, 1 0ther)	Black	Hepato-toxicity	grade 3 (5-10 times ULN) or grade 4 (> 10 times ULN) ALT elevations, with or without associated symptoms	hepatotoxicity	*B*58:01*	12	51	9	102	NA	NA	after multivariate analysis
						*DRB1*01:02*	8	51	4	102	NA	NA	after multivariate analysis
						*DRB1*01*	8	54	7	103	NA	NA	after univariate analysis
(Vitezica *et al.*, 2008)	French Caucasian	Caucasian	skin rash	NA	cADRs	*DRB1*01*	5	6	1	15	NA	NA	NVP/EFV hypersensitive patients

Appendix 6.1: continued

Study	Ethnicity or study location	New ethnicity	Phenotype	Phenotype definition	New phenotype	Reported HLA-alleles	# of cases		# of tolerant controls		# of population controls		Comments
							HLA pos	Total	HLA pos	Total	HLA pos	Total	
(YUAN *et al.*, 2011)	Taiwan	Northeast Asian	cADRs	severe cutaneous toxicity (grade 3 or 4); symptomatic grade >3 hepatic ALT/AST elevation (> 5 times ULN); or hepatic liver failure	cADRs	*C*04*	7	19	9	54	NA	NA	
	Thai	Thai					18	52	32	179	NA	NA	
	Black	Black African					15	27	15	77	NA	NA	
	White	Caucasian					26	77	58	277	NA	NA	
	Taiwan	Northeast Asian				*B*35*	4	19	8	54	NA	NA	
	Thai	Thai					10	52	7	173	NA	NA	
	Black	Black African					3	27	10	77	NA	NA	
	White	Caucasian					21	77	48	277	NA	NA	
	Taiwan	Northeast Asian	hepatic ADRs		hepatotoxicity	*DRB1*01*	1	30	1	233	NA	NA	
	Black	Black African					2	14	8	77	NA	NA	
	White	Caucasian					25	57	57	277	NA	NA	
	Taiwan	Northeast Asian	cADRs		cADRs	*B*35-C*04* haplo-type	4	19	0	54	NA	NA	
	Thai	Thai					10	52	3	173	NA	NA	
	Black	Black African					2	27	5	77	NA	NA	
	White	Caucasian					17	77	42	277	NA	NA	

Appendix 6.1: continued

Study	Ethnicity or study location	New ethnicity	Phenotype	Phenotype definition	New phenotype	Reported HLA-alleles	# of cases		# of tolerant controls		# of population controls		Comments
							HLA pos	Total	HLA pos	Total	HLA pos	Total	
Excluded as only allele frequency available; data set not received													
Likanonsakul et al., 2009	Thai	Thai	Skin rash	apparent skin rash without liver toxicity	cADRs	*C*08*	AF	39	AF	60	NA	NA	not significant association
(Littera *et al.*, 2006)	Sardinian	Caucasian	Hypersensitive	extensive skin rash, vesicular bullous or scaling skin lesions or skin manifestations combined with one or more of the following: fever, myalgia, arthralgia, visceral impairment, liver toxicity defined as grade 3 hepatotoxicity with AST/ALT > 5 times ULN and/or important signs of cholestasis	general hypersensitivity	*C*08*	AF	13	AF	36	AF	82	
						*B*14*	AF	13	AF	36	AF	82	
						*B*14-C*08* haplo-type	AF	13	AF	36	AF	82	
						*DRB1*01:01*	AF	13	AF	36	AF	82	not significant allele
						*DRB1*01:02*	AF	13	AF	36	AF	82	not significant allele
(Umapathy *et al.*, 2011)	India	Indian	skin rash	NA	cADRs	*B*35*	AF	40	40	NA	NA	3.38	

Appendix 6.1: continued

Study	Ethnicity or study location	New ethnicity	Phenotype	Phenotype definition	New phenotype	Reported HLA-alleles	# of cases		# of tolerant controls		# of population controls		Comments
							HLA pos	Total	HLA pos	Total	HLA pos	Total	
Excluded as only dataset to investigate this association													
(GATANAGA *et al.*, 2007)	Japanese (309/326)	Northeast Asian	Hypersensitive	11 patients with extensive skin rash (with fever in three) and one patient with Hep C who developed hepatotoxicity with AST/ALT > 3 times ULN	general hypersensitivity	*C*08*	5	12	3	29	NA	NA	

cADRs cutaneous adverse drug reactions, *DILI* drug induced liver injury, *HLA* human leukocyte antigen, *HSS* hypersensitivity syndrome, *NA* data not available, *NIR* nevirapine-induced rash, *SJS* Stevens-Johnson syndrome, *TEN* toxic epidermal necrolysis

Bibliography

ABRAHAM, L. J., LEELAYUWAT, C., GRIMSLEY, G., DEGLI-ESPOSTI, M. A., MANN, A., ZHANG, W. J., CHRISTIANSEN, F. T. & DAWKINS, R. L. 1992. Sequence differences between HLA-B and TNF distinguish different MHC ancestral haplotypes. *Tissue Antigens,* 39**,** 117-21.

ACUNA, G., FOERNZLER, D., LEONG, D., RABBIA, M., SMIT, R., DORFLINGER, E., GASSER, R., HOH, J., OTT, J., BORRONI, E.*, et al.* 2002. Pharmacogenetic analysis of adverse drug effect reveals genetic variant for susceptibility to liver toxicity. *Pharmacogenomics J,* 2**,** 327-34.

ADAM, J., PICHLER, W. J. & YERLY, D. 2011. Delayed drug hypersensitivity: models of T-cell stimulation. *Br J Clin Pharmacol,* 71**,** 701-7.

AGRAWAL, L., JIN, Q., ALTENBURG, J., MEYER, L., TUBIANA, R., THEODOROU, I. & ALKHATIB, G. 2007. CCR5Delta32 protein expression and stability are critical for resistance to human immunodeficiency virus type 1 in vivo. *J Virol,* 81**,** 8041-9.

AGRAWAL, L., LU, X., QINGWEN, J., VANHORN-ALI, Z., NICOLESCU, I. V., MCDERMOTT, D. H., MURPHY, P. M. & ALKHATIB, G. 2004. Role for CCR5Delta32 protein in resistance to R5, R5X4, and X4 human immunodeficiency virus type 1 in primary CD4+ cells. *J Virol,* 78**,** 2277-87.

AITHAL, G. P., WATKINS, P. B., ANDRADE, R. J., LARREY, D., MOLOKHIA, M., TAKIKAWA, H., HUNT, C. M., WILKE, R. A., AVIGAN, M., KAPLOWITZ, N.*, et al.* 2011. Case definition and phenotype standardization in drug-induced liver injury. *Clin Pharmacol Ther,* 89**,** 806-15.

ALBERTS, B., WILSON, J. H. & HUNT, T. 2008. *Molecular biology of the cell,* New York, Garland Science.

ALTFELD, M., ADDO, M. M., ROSENBERG, E. S., HECHT, F. M., LEE, P. K., VOGEL, M., YU, X. G., DRAENERT, R., JOHNSTON, M. N., STRICK, D.*, et al.* 2003. Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. *AIDS,* 17**,** 2581-91.

AMACHER, D. E. 2012. The primary role of hepatic metabolism in idiosyncratic drug-induced liver injury. *Expert Opin Drug Metab Toxicol,* 8**,** 335-47.

ANDERSEN, C. L., JENSEN, J. L. & ORNTOFT, T. F. 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res,* 64**,** 5245-50.

ANTOINE, D. J., DEAR, J. W., LEWIS, P. S., PLATT, V., COYLE, J., MASSON, M., THANACOODY, R. H., GRAY, A. J., WEBB, D. J., MOGGS, J. G.*, et al.* 2013. Mechanistic biomarkers provide early and sensitive detection of acetaminophen-induced acute liver injury at first presentation to hospital. *Hepatology,* 58**,** 777-787.

APPS, R., QI, Y., CARLSON, J. M., CHEN, H., GAO, X., THOMAS, R., YUKI, Y., DEL PRETE, G. Q., GOULDER, P., BRUMME, Z. L.*, et al.* 2013. Influence of HLA-C expression level on HIV control. *Science,* 340**,** 87-91.

ARELLANO, F. & SACRISTAN, J. A. 1993. Allopurinol hypersensitivity syndrome: a review. *Ann Pharmacother,* 27**,** 337-43.

ARONSON, J. K. & FERNER, R. E. 2003. Joining the DoTS: new approach to classifying adverse drug reactions. *BMJ,* 327**,** 1222-5.

ASUMALAHTI, K., VEAL, C., LAITINEN, T., SUOMELA, S., ALLEN, M., ELOMAA, O., MOSER, M., DE CID, R., RIPATTI, S., VORECHOVSKY, I.*, et al.* 2002. Coding haplotype analysis supports HCR as the putative susceptibility gene for psoriasis at the MHC PSORS1 locus. *Hum Mol Genet,* 11**,** 589-97.

BANNISTER, W. P., FRIIS-MOLLER, N., MOCROFT, A., VIARD, J. P., VAN LUNZEN, J., KIRK, O., GARGALIANOS, P., BANHEGYI, D., CHIESI, A., LUNDGREN, J. D.*, et al.* 2008. Incidence of abacavir hypersensitivity reactions in euroSIDA. *Antivir Ther,* 13**,** 687-96.

BARNER, A. & MYERS, M. 1998. Nevirapine and rashes. *Lancet,* 351**,** 1133.

BARRE-SINOUSSI, F. 1996. HIV as the cause of AIDS. *Lancet,* 348**,** 31-5.

BARRE-SINOUSSI, F., CHERMANN, J. C., REY, F., NUGEYRE, M. T., CHAMARET, S., GRUEST, J., DAUGUET, C., AXLER-BLIN, C., VEZINET-BRUN, F., ROUZIOUX, C.*, et al.* 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science,* 220**,** 868-71.

BARRETT, J. C., FRY, B., MALLER, J. & DALY, M. J. 2005. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics,* 21**,** 263-5.

BAUER, S., ABDGAWAD, M., GUNNARSSON, L., SEGELMARK, M., TAPPER, H. & HELLMARK, T. 2007. Proteinase 3 and CD177 are expressed on the plasma membrane of the same subset of neutrophils. *J Leukoc Biol,* 81**,** 458-64.

BAYAT, B., WERTH, S., SACHS, U. J., NEWMAN, D. K., NEWMAN, P. J. & SANTOSO, S. 2010. Neutrophil transmigration mediated by the neutrophil-specific antigen CD177 is influenced by the endothelial S536N dimorphism of platelet endothelial cell adhesion molecule-1. *J Immunol,* 184**,** 3889-96.

BECQUEMONT, L. 2009. Pharmacogenomics of adverse drug reactions: practical applications and perspectives. *Pharmacogenomics,* 10**,** 961-9.

BELLON, T., ALVAREZ, L., MAYORGA, C., MOREL, E., TORRES, M. J., MARTIN-DIAZ, M. A., DIAZ, R., RADIAL, A., CARBALLO, M. & BLANCA, M. 2010. Differential gene expression in drug hypersensitivity reactions: induction of alarmins in severe bullous diseases. *Br J Dermatol,* 162**,** 1014-22.

BERSOFF-MATCHA, S. J., MILLER, W. C., ABERG, J. A., VAN DER HORST, C., HAMRICK JR, H. J., POWDERLY, W. G. & MUNDY, L. M. 2001. Sex differences in nevirapine rash. *Clin Infect Dis,* 32**,** 124-9.

BETTINOTTI, M. P., OLSEN, A. & STRONCEK, D. 2002. The use of bioinformatics to identify the genomic structure of the gene that encodes neutrophil antigen NB1, CD177. *Clin Immunol,* 102**,** 138-44.

BHARADWAJ, M., ILLING, P., THEODOSSIS, A., PURCELL, A. W., ROSSJOHN, J. & MCCLUSKEY, J. 2012. Drug hypersensitivity and human leukocyte antigens of the major histocompatibility complex. *Annu Rev Pharmacol Toxicol,* 52**,** 401-31.

BHATNAGAR, N., LI, X., PADI, S. K., ZHANG, Q., TANG, M. S. & GUO, B. 2010. Downregulation of miR-205 and miR-31 confers resistance to chemotherapy-induced apoptosis in prostate cancer cells. *Cell Death Dis,* 1**,** e105.

BIANCHI, M. E. 2007. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol,* 81**,** 1-5.

BIGBY, M. 2001. Rates of cutaneous reactions to drugs. *Arch Dermatol,* 137**,** 765-70.

BOEHRINGER-INGELHEIM 2012. Virmaune Prescription Information. Ridgefield, CT, USA Boehringer Ingelheim Pharmaceuticals, Inc. .

BORREGAARD, N. 2010. Neutrophils, from marrow to microbes. *Immunity,* 33**,** 657-70.

BROWN, K. C., HOSSEINIPOUR, M. C., HOSKINS, J. M., THIRUMARAN, R. K., TIEN, H. C., WEIGEL, R., TAUZIE, J., SHUMBA, I., LAMBA, J. K., SCHUETZ, E. G.*, et al.* 2012. Exploration of CYP450 and drug transporter genotypes and correlations with nevirapine exposure in Malawians. *Pharmacogenomics,* 13**,** 113-21.

BURNET, F. M. 1961. Immunological recognition of self. *Science,* 133**,** 307-11.

BUX, J., BECKER, F., SEEGER, W., KILPATRICK, D., CHAPMAN, J. & WATERS, A. 1996. Transfusion-related acute lung injury due to HLA-A2-specific antibodies in recipient and NB1-specific antibodies in donor blood. *Br J Haematol,* 93**,** 707-13.

CAMPBELL, P. J., SCOTT, L. M., BUCK, G., WHEATLEY, K., EAST, C. L., MARSDEN, J. T., DUFFY, A., BOYD, E. M., BENCH, A. J., SCOTT, M. A.*, et al.* 2005. Definition of subtypes of essential thrombocythaemia and relation to polycythaemia vera based on JAK2 V617F mutation status: a prospective study. *Lancet,* 366**,** 1945-53.

CAO, K., HOLLENBACH, J., SHI, X., SHI, W., CHOPEK, M. & FERNANDEZ-VINA, M. A. 2001. Analysis of the frequencies of HLA-A, B, and C alleles and haplotypes in the five major ethnic groups of the United States reveals high levels of diversity in these loci and contrasting distribution patterns in these populations. *Hum Immunol,* 62**,** 1009-30.

CAO, Y., QIN, L., ZHANG, L., SAFRIT, J. & HO, D. D. 1995. Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *N Engl J Med,* 332**,** 201-8.

CAPRONI, M., TORCHIA, D., SCHINCAGLIA, E., VOLPI, W., FREZZOLINI, A., SCHENA, D., MARZANO, A., QUAGLINO, P., DE SIMONE, C., PARODI, A.*, et al.* 2006. The CD40/CD40 ligand system is expressed in the cutaneous lesions of erythema multiforme and Stevens-Johnson syndrome/toxic epidermal necrolysis spectrum. *Br J Dermatol,* 154**,** 319-24.

CARR, D. F., CHAPONDA, M., JORGENSEN, A. L., CASTRO, E. C., VAN OOSTERHOUT, J. J., KHOO, S. H., LALLOO, D. G., HEYDERMAN, R. S., ALFIREVIC, A. & PIRMOHAMED, M. 2013. Association of Human Leukocyte Antigen Alleles and Nevirapine Hypersensitivity in a Malawian HIV-Infected Population. *Clin Infect Dis,* 56**,** 1330-9.

CARRINGTON, M., NELSON, G. W., MARTIN, M. P., KISSNER, T., VLAHOV, D., GOEDERT, J. J., KASLOW, R., BUCHBINDER, S., HOOTS, K. & O'BRIEN, S. J. 1999. HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. *Science,* 283**,** 1748-52.

CARUCCIO, L., BETTINOTTI, M., DIRECTOR-MYSKA, A. E., ARTHUR, D. C. & STRONCEK, D. 2006. The gene overexpressed in polycythemia rubra vera, PRV-1, and the gene encoding a neutrophil alloantigen, NB1, are alleles of a single gene, CD177, in chromosome band 19q13.31. *Transfusion,* 46**,** 441-7.

CARUCCIO, L., BETTINOTTI, M., MATSUO, K., SHARON, V. & STRONCEK, D. 2003. Expression of human neutrophil antigen-2a (NB1) is increased in pregnancy. *Transfusion,* 43**,** 357-63.

CARUCCIO, L., WALKOVICH, K., BETTINOTTI, M., SCHULLER, R. & STRONCEK, D. 2004. CD177 polymorphisms: correlation between high-frequency single nucleotide polymorphisms and neutrophil surface protein expression. *Transfusion,* 44**,** 77-82.

CENTRAL INTELLIGENCE AGENCY. 2009. *The World Factbook - Country Comparison: HIV/AIDS - Adult Prevalence Rate* [Online]. Available: https://www.cia.gov/library/publications/the-world-factbook/rankorder/2155rank.html?countryname=Malawi&countrycode=mi&regionCode=afr&rank=9#mi [Accessed October 2013].

CERMELLI, S., RUGGIERI, A., MARRERO, J. A., IOANNOU, G. N. & BERETTA, L. 2011. Circulating MicroRNAs in Patients with Chronic Hepatitis C and Non-Alcoholic Fatty Liver Disease. *PLoS ONE,* 6**,** e23937.

CHALASANI, N. & BJORNSSON, E. 2010. Risk factors for idiosyncratic drug-induced liver injury. *Gastroenterology,* 138**,** 2246-59.

CHAN, M., LIAW, C. S., JI, S. M., TAN, H. H., WONG, C. Y., THIKE, A. A., TAN, P. H., HO, G. H. & LEE, A. S. 2013. Identification of circulating microRNA signatures for breast cancer detection. *Clin Cancer Res,* 19**,** 4477-87.

CHANG, C. C., TOO, C. L., MURAD, S. & HUSSEIN, S. H. 2011. Association of HLA-B*1502 allele with carbamazepine-induced toxic epidermal necrolysis and Stevens-Johnson syndrome in the multi-ethnic Malaysian population. *Int J Dermatol,* 50**,** 221-4.

CHANTARANGSU, S., MUSHIRODA, T., MAHASIRIMONGKOL, S., KIERTIBURANAKUL, S., SUNGKANUPARPH, S., MANOSUTHI, W., TANTISIRIWAT, W., CHAROENYINGWATTANA, A., SURA, T., CHANTRATITA, W.*, et al.* 2009. HLA-B*3505 allele is a strong predictor for nevirapine-induced skin adverse drug reactions in HIV-infected Thai patients. *Pharmacogenet Genomics,* 19**,** 139-46.

CHANTARANGSU, S., MUSHIRODA, T., MAHASIRIMONGKOL, S., KIERTIBURANAKUL, S., SUNGKANUPARPH, S., MANOSUTHI, W., TANTISIRIWAT, W., CHAROENYINGWATTANA, A.,

SURA, T., TAKAHASHI, A., *et al.* 2011. Genome-wide association study identifies variations in 6p21.3 associated with nevirapine-induced rash. *Clin Infect Dis,* 53, 341-8.

CHEESEMAN, S. H., HATTOX, S. E., MCLAUGHLIN, M. M., KOUP, R. A., ANDREWS, C., BOVA, C. A., PAV, J. W., ROY, T., SULLIVAN, J. L. & KEIRNS, J. J. 1993. Pharmacokinetics of nevirapine: initial single-rising-dose study in humans. *Antimicrob Agents Chemother,* 37, 178-82.

CHEN, J., MANNARGUDI, B. M., XU, L. & UETRECHT, J. 2008a. Demonstration of the metabolic pathway responsible for nevirapine-induced skin rash. *Chem Res Toxicol,* 21, 1862-70.

CHEN, P., LIN, J. J., LU, C. S., ONG, C. T., HSIEH, P. F., YANG, C. C., TAI, C. T., WU, S. L., LU, C. H., HSU, Y. C., *et al.* 2011. Carbamazepine-induced toxic effects and HLA-B*1502 screening in Taiwan. *N Engl J Med,* 364, 1126-33.

CHEN, X., BA, Y., MA, L., CAI, X., YIN, Y., WANG, K., GUO, J., ZHANG, Y., CHEN, J., GUO, X., *et al.* 2008b. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res,* 18, 997-1006.

CHEN, X., THARMANATHAN, T., MANNARGUDI, B., GOU, H. & UETRECHT, J. P. 2009. A study of the specificity of lymphocytes in nevirapine-induced skin rash. *J Pharmacol Exp Ther,* 331, 836-41.

CHERMANN, J. C., BARRE-SINOUSSI, F., DAUGUET, C., BRUN-VEZINET, F., ROUZIOUX, C., ROZENBAUM, W. & MONTAGNIER, L. 1983. Isolation of a new retrovirus in a patient at risk for acquired immunodeficiency syndrome. *Antibiot Chemother (1971),* 32, 48-53.

CHESSMAN, D., KOSTENKO, L., LETHBORG, T., PURCELL, A. W., WILLIAMSON, N. A., CHEN, Z., KJER-NIELSEN, L., MIFSUD, N. A., TAIT, B. D., HOLDSWORTH, R., *et al.* 2008. Human leukocyte antigen class I-restricted activation of CD8+ T cells provides the immunogenetic basis of a systemic drug hypersensitivity. *Immunity,* 28, 822-32.

CHOU, M., BERTRAND, J., SEGERAL, O., VERSTUYFT, C., BORAND, L., COMETS, E., LE TIEC, C., BECQUEMONT, L., OUK, V., MENTRE, F., *et al.* 2010. Population pharmacokinetic-pharmacogenetic study of nevirapine in HIV-infected Cambodian patients. *Antimicrob Agents Chemother,* 54, 4432-9.

CHUNG, W. H., HUNG, S. I., HONG, H. S., HSIH, M. S., YANG, L. C., HO, H. C., WU, J. Y. & CHEN, Y. T. 2004. Medical genetics: a marker for Stevens-Johnson syndrome. *Nature,* 428, 486.

CICCACCI, C., BORGIANI, P., CEFFA, S., SIRIANNI, E., MARAZZI, M. C., ALTAN, A. M., PATURZO, G., BRAMANTI, P., NOVELLI, G. & PALOMBI, L. 2010. Nevirapine-induced hepatotoxicity and pharmacogenetics: a retrospective study in a population from Mozambique. *Pharmacogenomics,* 11, 23-31.

CICCACCI, C., DI FUSCO, D., MARAZZI, M. C., ZIMBA, I., ERBA, F., NOVELLI, G., PALOMBI, L., BORGIANI, P. & LIOTTA, G. 2013. Association between CYP2B6 polymorphisms and Nevirapine-induced SJS/TEN: a pharmacogenetics study. *Eur J Clin Pharmacol*.

CLAVEL, F., GUETARD, D., BRUN-VEZINET, F., CHAMARET, S., REY, M. A., SANTOS-FERREIRA, M. O., LAURENT, A. G., DAUGUET, C., KATLAMA, C., ROUZIOUX, C., *et al.* 1986. Isolation of a new human retrovirus from West African patients with AIDS. *Science,* 233, 343-6.

COBOS, A., SANCHEZ, P., AGUADO, J. & CARRASCO, J. L. 2011. Methodological quality in pharmacogenetic studies with binary assessment of treatment response: a review. *Pharmacogenet Genomics,* 21, 243-50.

COOPER, C. L. & VAN HEESWIJK, R. P. 2007. Once-daily nevirapine dosing: a pharmacokinetics, efficacy and safety review. *HIV Med,* 8, 1-7.

COSTELLO, C., TANG, J., RIVERS, C., KARITA, E., MEIZEN-DERR, J., ALLEN, S. & KASLOW, R. A. 1999. HLA-B*5703 independently associated with slower HIV-1 disease progression in Rwandan women. *AIDS,* 13, 1990-1.

CREEMERS, E. E., TIJSEN, A. J. & PINTO, Y. M. 2012. Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? *Circ Res,* 110**,** 483-95.

CUTRELL, A. G., HERNANDEZ, J. E., FLEMING, J. W., EDWARDS, M. T., MOORE, M. A., BROTHERS, C. H. & SCOTT, T. R. 2004. Updated clinical risk factor analysis of suspected hypersensitivity reactions to abacavir. *Ann Pharmacother,* 38**,** 2171-2.

DAINICHI, T., UCHI, H., MOROI, Y. & FURUE, M. 2007. Stevens-Johnson syndrome, drug-induced hypersensitivity syndrome and toxic epidermal necrolysis caused by allopurinol in patients with a common HLA allele: what causes the diversity? *Dermatology,* 215**,** 86-8.

DALY, A. K., DONALDSON, P. T., BHATNAGAR, P., SHEN, Y., PE'ER, I., FLORATOS, A., DALY, M. J., GOLDSTEIN, D. B., JOHN, S., NELSON, M. R.*, et al.* 2009. HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. *Nat Genet,* 41**,** 816-9.

DE BAKKER, P. I., YELENSKY, R., PE'ER, I., GABRIEL, S. B., DALY, M. J. & ALTSHULER, D. 2005. Efficiency and power in genetic association studies. *Nat Genet,* 37**,** 1217-23.

DE CLERCQ, E. 2009. Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV. *Int J Antimicrob Agents,* 33**,** 307-20.

DEAN, M., CARRINGTON, M., WINKLER, C., HUTTLEY, G. A., SMITH, M. W., ALLIKMETS, R., GOEDERT, J. J., BUCHBINDER, S. P., VITTINGHOFF, E., GOMPERTS, E.*, et al.* 1996. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science,* 273**,** 1856-62.

DEEKS, S. G. 2001. International perspectives on antiretroviral resistance. Nonnucleoside reverse transcriptase inhibitor resistance. *J Acquir Immune Defic Syndr,* 26 Suppl 1**,** S25-33.

DEPARTMENT FOR HIV AND AIDS 2012. Integrated HIV Program Report. Malawi: Government of Malawi, Ministry of Health.

DESCAMPS, V., BOUSCARAT, F., LAGLENNE, S., ASLANGUL, E., VEBER, B., DESCAMPS, D., SARAUX, J. L., GRANGE, M. J., GROSSIN, M., NAVRATIL, E.*, et al.* 1997. Human herpesvirus 6 infection associated with anticonvulsant hypersensitivity syndrome and reactive haemophagocytic syndrome. *Br J Dermatol,* 137**,** 605-8.

DICKINSON, L., CHAPONDA, M., CARR, D. F., VAN OOSTERHOUT, J. J., KUMWENDA, J., LALLOO, D. G., PIRMOHAMED, M., HEYDERMAN, R. S. & KHOO, S. H. 2013. Population Pharmacokinetic and Pharmacogenetic Analysis of Nevirapine in Hypersensitive and Tolerant HIV-Infected Patients from Malawi. AAC (submitted).

DIETERICH, D. T., ROBINSON, P. A., LOVE, J. & STERN, J. O. 2004. Drug-induced liver injury associated with the use of nonnucleoside reverse-transcriptase inhibitors. *Clin Infect Dis,* 38 Suppl 2**,** S80-9.

DIETRICH, C. G., GEIER, A. & OUDE ELFERINK, R. P. 2003. ABC of oral bioavailability: transporters as gatekeepers in the gut. *Gut,* 52**,** 1788-95.

DIEZ, R. A. 1990. HLA-B27 and agranulocytosis by levamisole. *Immunol Today,* 11**,** 270.

DIVISION OF AIDS, R. S. C. *Safety and Pharmacovigilance, Grading Tables* [Online]. National Institue of Allergy and Infectious Diseases. Available: http://rsc.tech-res.com/safetyandpharmacovigilance/gradingtables.aspx [Accessed December 2013].

EDWARDS, I. R. & ARONSON, J. K. 2000. Adverse drug reactions: definitions, diagnosis, and management. *Lancet,* 356**,** 1255-9.

ENRIGHT, A. J., JOHN, B., GAUL, U., TUSCHL, T., SANDER, C. & MARKS, D. S. 2003. MicroRNA targets in Drosophila. *Genome Biol,* 5**,** R1.

ERICKSON, D. A., MATHER, G., TRAGER, W. F., LEVY, R. H. & KEIRNS, J. J. 1999. Characterization of the in vitro biotransformation of the HIV-1 reverse

transcriptase inhibitor nevirapine by human hepatic cytochromes P-450. *Drug Metab Dispos,* 27**,** 1488-95.

EU 2010. Directive 2010/84/EU of the European Parliament and of the Council. *In:* UNION, E. P. A. T. C. O. T. E. (ed.). Strasbourg: European Union.

EVANS, W. E., HORNER, M., CHU, Y. Q., KALWINSKY, D. & ROBERTS, W. M. 1991. Altered mercaptopurine metabolism, toxic effects, and dosage requirement in a thiopurine methyltransferase-deficient child with acute lymphocytic leukemia. *J Pediatr,* 119**,** 985-9.

EVANS, W. E. & MCLEOD, H. L. 2003. Pharmacogenomics--drug disposition, drug targets, and side effects. *N Engl J Med,* 348**,** 538-49.

FABBRI, M. 2013. MicroRNAs and cancer: towards a personalized medicine. *Curr Mol Med,* 13**,** 751-6.

FAGOT, J. P., MOCKENHAUPT, M., BOUWES-BAVINCK, J. N., NALDI, L., VIBOUD, C., ROUJEAU, J. C. & EURO, S. S. G. 2001. Nevirapine and the risk of Stevens-Johnson syndrome or toxic epidermal necrolysis. *AIDS,* 15**,** 1843-8.

FARAZI, T. A., SPITZER, J. I., MOROZOV, P. & TUSCHL, T. 2011. miRNAs in human cancer. *J Pathol,* 223**,** 102-15.

FDA. 2007. *Information for Healthcare Professionals: Dangerous or Even Fatal Skin Reactions - Carbamazepine (marketed as Carbatrol, Equetro, Tegretol, and generics* [Online]. Silver Spring, MD, USA: U.S. Food and Drug Administration, U.S. Department of Health and Human Services. Available: http://www.fda.gov/Drugs/DrugSafety/PostmarketdrugsafetyinformationforPatientsandProviders/ucm124718.htm [Accessed 01/10 2013].

FDA. 2008. *Information for Healthcare Professionals: Abacavir (marketed as Ziagen) and Abacavir-Containing Medications* [Online]. Silver Spring, MD, USA: U.S. Food and Drug Administration, U.S. Department of Health and Human Services. Available: http://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/ucm123927.htm [Accessed 27/09 2013].

FDA. 2013. *Antiretroviral drugs used in the treatment of HIV infection* [Online]. U.S. Food and Drug Administration. Available: http://www.fda.gov/ForConsumers/byAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm118915.htm [Accessed November 2013].

FOELL, D., WITTKOWSKI, H. & ROTH, J. 2007. Mechanisms of disease: a 'DAMP' view of inflammatory arthritis. *Nat Clin Pract Rheumatol,* 3**,** 382-90.

FREED, E. O. 2001. HIV-1 replication. *Somat Cell Mol Genet,* 26**,** 13-33.

GANGAR, M., ARIAS, G., O'BRIEN, J. G. & KEMPER, C. A. 2000. Frequency of cutaneous reactions on rechallenge with nevirapine and delavirdine. *Ann Pharmacother,* 34**,** 839-42.

GAO, S., GUI, X. E., LIANG, K., LIU, Z., HU, J. & DONG, B. 2012. HLA-dependent hypersensitivity reaction to nevirapine in Chinese Han HIV-infected patients. *AIDS Res Hum Retroviruses,* 28**,** 540-3.

GASCHE, Y., DAALI, Y., FATHI, M., CHIAPPE, A., COTTINI, S., DAYER, P. & DESMEULES, J. 2004. Codeine intoxication associated with ultrarapid CYP2D6 metabolism. *N Engl J Med,* 351**,** 2827-31.

GATANAGA, H., YAZAKI, H., TANUMA, J., HONDA, M., GENKA, I., TERUYA, K., TACHIKAWA, N., KIKUCHI, Y. & OKA, S. 2007. HLA-Cw8 primarily associated with hypersensitivity to nevirapine. *AIDS,* 21**,** 264-5.

GELL, P. G. H. & COOMBS, R. R. A. 1968. *Clinical aspects of immunology,* Oxford, Edinburgh,, Blackwell Scientific.

GENOMES PROJECT CONSORTIUM, ABECASIS, G. R., AUTON, A., BROOKS, L. D., DEPRISTO, M. A., DURBIN, R. M., HANDSAKER, R. E., KANG, H. M., MARTH, G. T. & MCVEAN, G. A. 2012. An integrated map of genetic variation from 1,092 human genomes. *Nature,* 491**,** 56-65.

GOHRING, K., WOLFF, J., DOPPL, W., SCHMIDT, K. L., FENCHEL, K., PRALLE, H., SIBELIUS, U. & BUX, J. 2004. Neutrophil CD177 (NB1 gp, HNA-2a) expression is increased in severe bacterial infections and polycythaemia vera. *Br J Haematol,* 126**,** 252-4.

GOLDSCHMEDING, R., VANDALEN, C. M., FABER, N., CALAFAT, J., HUIZINGA, T. W. J., VANDERSCHOOT, C. E., CLEMENT, L. T. & VONDEMBORNE, A. E. G. K. 1992. Further Characterization of the Nb-1 Antigen as a Variably Expressed 56-62 Kd Gpi-Linked Glycoprotein of Plasma-Membranes and Specific Granules of Neutrophils. *British Journal of Haematology,* 81**,** 336-345.

GONZALEZ DE REQUENA, D., BONORA, S., GARAZZINO, S., SCIANDRA, M., D'AVOLIO, A., RAITERI, R., MARRONE, R., BOFFITO, M., DE ROSA, F. G., SINICCO, A.*, et al.* 2005. Nevirapine plasma exposure affects both durability of viral suppression and selection of nevirapine primary resistance mutations in a clinical setting. *Antimicrob Agents Chemother,* 49**,** 3966-9.

GONZALEZ-GALARZA, F. F., CHRISTMAS, S., MIDDLETON, D. & JONES, A. R. 2011. *Allele frequency net: a database and online repository for immune gene frequencies in worldwide populations* [Online]. Available: http://www.allelefrequencies.net [Accessed 01/10 2013].

GOTTLIEB, M. S., SCHROFF, R., SCHANKER, H. M., WEISMAN, J. D., FAN, P. T., WOLF, R. A. & SAXON, A. 1981. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *N Engl J Med,* 305**,** 1425-31.

GOZALO, C., GERARD, L., LOISEAU, P., MORAND-JOUBERT, L., PEYTAVIN, G., MOLINA, J. M., DELLAMONICA, P., BECQUEMONT, L., ABOULKER, J. P., LAUNAY, O.*, et al.* 2011. Pharmacogenetics of toxicity, plasma trough concentration and treatment outcome with nevirapine-containing regimen in anti-retroviral-naive HIV-infected adults: an exploratory study of the TRIANON ANRS 081 trial. *Basic Clin Pharmacol Toxicol,* 109**,** 513-20.

GRIFFITHS-JONES, S. 2006. miRBase: the microRNA sequence database. *Methods Mol Biol,* 342**,** 129-38.

HAAS, D. W., BARTLETT, J. A., ANDERSEN, J. W., SANNE, I., WILKINSON, G. R., HINKLE, J., ROUSSEAU, F., INGRAM, C. D., SHAW, A., LEDERMAN, M. M.*, et al.* 2006. Pharmacogenetics of nevirapine-associated hepatotoxicity: an Adult AIDS Clinical Trials Group collaboration. *Clin Infect Dis,* 43**,** 783-6.

HALL, D. B. & MACGREGOR, T. R. 2007. Case-control exploration of relationships between early rash or liver toxicity and plasma concentrations of nevirapine and primary metabolites. *HIV Clin Trials,* 8**,** 391-9.

HAMMER, S. M., ERON, J. J., Jr., REISS, P., SCHOOLEY, R. T., THOMPSON, M. A., WALMSLEY, S., CAHN, P., FISCHL, M. A., GATELL, J. M., HIRSCH, M. S.*, et al.* 2008. Antiretroviral treatment of adult HIV infection: 2008 recommendations of the International AIDS Society-USA panel. *JAMA,* 300**,** 555-70.

HARI, Y., FRUTIG-SCHNYDER, K., HURNI, M., YAWALKAR, N., ZANNI, M. P., SCHNYDER, B., KAPPELER, A., VON GREYERZ, S., BRAATHEN, L. R. & PICHLER, W. J. 2001. T cell involvement in cutaneous drug eruptions. *Clin Exp Allergy,* 31**,** 1398-408.

HASHIZUME, H., TAKIGAWA, M. & TOKURA, Y. 2002. Characterization of drug-specific T cells in phenobarbital-induced eruption. *J Immunol,* 168**,** 5359-68.

HAUSER, I. A., SCHAEFFELER, E., GAUER, S., SCHEUERMANN, E. H., WEGNER, B., GOSSMANN, J., ACKERMANN, H., SEIDL, C., HOCHER, B., ZANGER, U. M.*, et al.* 2005. ABCB1 genotype of the donor but not of the recipient is a major risk factor for cyclosporine-related nephrotoxicity after renal transplantation. *J Am Soc Nephrol,* 16**,** 1501-11.

HAUSMANN, O., SCHNYDER, B. & PICHLER, W. J. 2010. Drug hypersensitivity reactions involving skin. *Handb Exp Pharmacol***,** 29-55.

HAVLIR, D. V., EASTMAN, S., GAMST, A. & RICHMAN, D. D. 1996. Nevirapine-resistant human immunodeficiency virus: kinetics of replication and estimated prevalence in untreated patients. *J Virol,* 70**,** 7894-9.

HEIL, S. G., VAN DER ENDE, M. E., SCHENK, P. W., VAN DER HEIDEN, I., LINDEMANS, J., BURGER, D. & VAN SCHAIK, R. H. 2012. Associations between ABCB1, CYP2A6, CYP2B6, CYP2D6, and CYP3A5 alleles in relation to efavirenz and nevirapine pharmacokinetics in HIV-infected individuals. *Ther Drug Monit,* 34**,** 153-9.

HENDEL, H., CAILLAT-ZUCMAN, S., LEBUANEC, H., CARRINGTON, M., O'BRIEN, S., ANDRIEU, J. M., SCHACHTER, F., ZAGURY, D., RAPPAPORT, J., WINKLER, C.*, et al.* 1999. New class I and II HLA alleles strongly associated with opposite patterns of progression to AIDS. *J Immunol,* 162**,** 6942-6.

HETHERINGTON, S., HUGHES, A. R., MOSTELLER, M., SHORTINO, D., BAKER, K. L., SPREEN, W., LAI, E., DAVIES, K., HANDLEY, A., DOW, D. J.*, et al.* 2002. Genetic variations in HLA-B region and hypersensitivity reactions to abacavir. *Lancet,* 359**,** 1121-2.

HETHERINGTON, S., MCGUIRK, S., POWELL, G., CUTRELL, A., NADERER, O., SPREEN, B., LAFON, S., PEARCE, G. & STEEL, H. 2001. Hypersensitivity reactions during therapy with the nucleoside reverse transcriptase inhibitor abacavir. *Clin Ther,* 23**,** 1603-14.

HIGGINS, J. P. & SALLY, G. 2011. *Cochrane Handbook for Systematic Reviews of Interventions.* Version 5.1.0. The Cochrane Collaboration. http://handbook.cochrane.org/

HILL, A. V., ALLSOPP, C. E., KWIATKOWSKI, D., ANSTEY, N. M., TWUMASI, P., ROWE, P. A., BENNETT, S., BREWSTER, D., MCMICHAEL, A. J. & GREENWOOD, B. M. 1991. Common west African HLA antigens are associated with protection from severe malaria. *Nature,* 352**,** 595-600.

HU, N., WESTRA, J., HUITEMA, M. G., BIJL, M., BROUWER, E., STEGEMAN, C. A., HEERINGA, P., LIMBURG, P. C. & KALLENBERG, C. G. 2009. Coexpression of CD177 and membrane proteinase 3 on neutrophils in antineutrophil cytoplasmic autoantibody-associated systemic vasculitis: anti-proteinase 3-mediated neutrophil activation is independent of the role of CD177-expressing neutrophils. *Arthritis Rheum,* 60**,** 1548-57.

HUGHES, A. R., MOSTELLER, M., BANSAL, A. T., DAVIES, K., HANELINE, S. A., LAI, E. H., NANGLE, K., SCOTT, T., SPREEN, W. R., WARREN, L. L.*, et al.* 2004. Association of genetic variations in HLA-B region with hypersensitivity to abacavir in some, but not all, populations. *Pharmacogenomics,* 5**,** 203-11.

HUNG, S. I., CHUNG, W. H., LIOU, L. B., CHU, C. C., LIN, M., HUANG, H. P., LIN, Y. L., LAN, J. L., YANG, L. C., HONG, H. S.*, et al.* 2005. HLA-B*5801 allele as a genetic marker for severe cutaneous adverse reactions caused by allopurinol. *Proc Natl Acad Sci U S A,* 102**,** 4134-9.

HUNG, S. I., CHUNG, W. H., LIU, Z. S., CHEN, C. H., HSIH, M. S., HUI, R. C., CHU, C. Y. & CHEN, Y. T. 2010. Common risk allele in aromatic antiepileptic-drug induced Stevens-Johnson syndrome and toxic epidermal necrolysis in Han Chinese. *Pharmacogenomics,* 11**,** 349-56.

HUNZIKER, T., KUNZI, U. P., BRAUNSCHWEIG, S., ZEHNDER, D. & HOIGNE, R. 1997. Comprehensive hospital drug monitoring (CHDM): adverse skin reactions, a 20-year survey. *Allergy,* 52**,** 388-93.

IBM CORP., IBM SPSS Statistics for Mac OS X [Computer Program]. Version 20.0. Armonk, NY: IBM Corp., 2011.

ICHIHARA, A., WANG, Z., JINNIN, M., IZUNO, Y., SHIMOZONO, N., YAMANE, K., FUJISAWA, A., MORIYA, C., FUKUSHIMA, S., INOUE, Y.*, et al.* 2013. Upregulation of miR-18a-5p contributes to epidermal necrolysis in severe drug eruptions. *J Allergy Clin Immunol*.

ICKOVICS, J. R. & MEISLER, A. W. 1997. Adherence in AIDS clinical trials: a framework for clinical research and clinical care. *J Clin Epidemiol,* 50**,** 385-91.

IKEDA, H., TAKAHASHI, Y., YAMAZAKI, E., FUJIWARA, T., KANIWA, N., SAITO, Y., AIHARA, M., KASHIWAGI, M. & MURAMATSU, M. 2010. HLA class I markers in Japanese patients with carbamazepine-induced cutaneous adverse reactions. *Epilepsia,* 51**,** 297-300.

IKEDA, S., KONG, S. W., LU, J., BISPING, E., ZHANG, H., ALLEN, P. D., GOLUB, T. R., PIESKE, B. & PU, W. T. 2007. Altered microRNA expression in human heart disease. *Physiol Genomics,* 31**,** 367-73.

ILLING, P. T., VIVIAN, J. P., DUDEK, N. L., KOSTENKO, L., CHEN, Z., BHARADWAJ, M., MILES, J. J., KJER-NIELSEN, L., GRAS, S., WILLIAMSON, N. A.*, et al.* 2012. Immune self-reactivity triggered by drug-modified HLA-peptide repertoire. *Nature,* 486**,** 554-8.

INTERNATIONAL HAPMAP, C. 2003. The International HapMap Project. *Nature,* 426**,** 789-96.

INTERNATIONAL HIV CONTROLLERS STUDY, PEREYRA, F., JIA, X., MCLAREN, P. J., TELENTI, A., DE BAKKER, P. I., WALKER, B. D., RIPKE, S., BRUMME, C. J., PULIT, S. L.*, et al.* 2010. The major genetic determinants of HIV-1 control affect HLA class I peptide presentation. *Science,* 330**,** 1551-7.

ISHIKAWA, T. 1992. The ATP-dependent glutathione S-conjugate export pump. *Trends Biochem Sci,* 17**,** 463-8.

ITESCU, S., MATHUR-WAGH, U., SKOVRON, M. L., BRANCATO, L. J., MARMOR, M., ZELENIUCH-JACQUOTTE, A. & WINCHESTER, R. 1992. HLA-B35 is associated with accelerated progression to AIDS. *J Acquir Immune Defic Syndr,* 5**,** 37-45.

IYER, L., DAS, S., JANISCH, L., WEN, M., RAMIREZ, J., KARRISON, T., FLEMING, G. F., VOKES, E. E., SCHILSKY, R. L. & RATAIN, M. J. 2002. UGT1A1*28 polymorphism as a determinant of irinotecan disposition and toxicity. *Pharmacogenomics J,* 2**,** 43-7.

JAMES, C., UGO, V., LE COUEDIC, J. P., STAERK, J., DELHOMMEAU, F., LACOUT, C., GARCON, L., RASLOVA, H., BERGER, R., BENNACEUR-GRISCELLI, A.*, et al.* 2005. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature,* 434**,** 1144-8.

JANKOWSKA, A. M., SZPURKA, H., CALABRO, M., MOHAN, S., SCHADE, A. E., CLEMENTE, M., SILVERSTEIN, R. L. & MACIEJEWSKI, J. P. 2011. Loss of expression of neutrophil proteinase-3: a factor contributing to thrombotic risk in paroxysmal nocturnal hemoglobinuria. *Haematologica,* 96**,** 954-62.

JIANG, X. 2011. The emerging role of microRNAs in asthma. *Mol Cell Biochem,* 353**,** 35-40.

JOFFRE, O. P., SEGURA, E., SAVINA, A. & AMIGORENA, S. 2012. Cross-presentation by dendritic cells. *Nat Rev Immunol,* 12**,** 557-69.

JOHNSON, C. D., ESQUELA-KERSCHER, A., STEFANI, G., BYROM, M., KELNAR, K., OVCHARENKO, D., WILSON, M., WANG, X., SHELTON, J., SHINGARA, J.*, et al.* 2007. The let-7 microRNA represses cell proliferation pathways in human cells. *Cancer Res,* 67**,** 7713-22.

JORDAN, D. M., KIEZUN, A., BAXTER, S. M., AGARWALA, V., GREEN, R. C., MURRAY, M. F., PUGH, T., LEBO, M. S., REHM, H. L., FUNKE, B. H.*, et al.* 2011. Development and validation of a computational method for assessment of missense variants in hypertrophic cardiomyopathy. *Am J Hum Genet,* 88**,** 183-92.

JORGENSEN, A. L. & WILLIAMSON, P. R. 2008. Methodological quality of pharmacogenetic studies: issues of concern. *Stat Med,* 27**,** 6547-69.

JUNKER, A., KRUMBHOLZ, M., EISELE, S., MOHAN, H., AUGSTEIN, F., BITTNER, R., LASSMANN, H., WEKERLE, H., HOHLFELD, R. & MEINL, E. 2009. MicroRNA profiling of multiple sclerosis lesions identifies modulators of the regulatory protein CD47. *Brain,* 132**,** 3342-52.

KALLINGS, L. O. 2008. The first postmodern pandemic: 25 years of HIV/ AIDS. *J Intern Med,* 263**,** 218-43.

KANG, H. R., JEE, Y. K., KIM, Y. S., LEE, C. H., JUNG, J. W., KIM, S. H., PARK, H. W., CHANG, Y. S., JANG, I. J., CHO, S. H.*, et al.* 2011. Positive and negative associations of HLA class I

alleles with allopurinol-induced SCARs in Koreans. *Pharmacogenet Genomics,* 21**,** 303-7.

KANIWA, N., SAITO, Y., AIHARA, M., MATSUNAGA, K., TOHKIN, M., KUROSE, K., FURUYA, H., TAKAHASHI, Y., MURAMATSU, M., KINOSHITA, S.*, et al.* 2010. HLA-B*1511 is a risk factor for carbamazepine-induced Stevens-Johnson syndrome and toxic epidermal necrolysis in Japanese patients. *Epilepsia,* 51**,** 2461-5.

KANIWA, N., SAITO, Y., AIHARA, M., MATSUNAGA, K., TOHKIN, M., KUROSE, K., SAWADA, J., FURUYA, H., TAKAHASHI, Y., MURAMATSU, M.*, et al.* 2008. HLA-B locus in Japanese patients with anti-epileptics and allopurinol-related Stevens-Johnson syndrome and toxic epidermal necrolysis. *Pharmacogenomics,* 9**,** 1617-22.

KAPLOWITZ, N. 2001. Drug-induced liver disorders: implications for drug development and regulation. *Drug Saf,* 24**,** 483-90.

KAPLOWITZ, N. 2004. Drug-induced liver injury. *Clin Infect Dis,* 38 Suppl 2**,** S44-8.

KAPLOWITZ, N. 2005. Idiosyncratic drug hepatotoxicity. *Nat Rev Drug Discov,* 4**,** 489-99.

KASHIWAGI, M., AIHARA, M., TAKAHASHI, Y., YAMAZAKI, E., YAMANE, Y., SONG, Y., MURAMATSU, M. & IKEZAWA, Z. 2008. Human leukocyte antigen genotypes in carbamazepine-induced severe cutaneous adverse drug response in Japanese patients. *J Dermatol,* 35**,** 683-5.

KASLOW, R. A., CARRINGTON, M., APPLE, R., PARK, L., MUNOZ, A., SAAH, A. J., GOEDERT, J. J., WINKLER, C., O'BRIEN, S. J., RINALDO, C.*, et al.* 1996. Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat Med,* 2**,** 405-11.

KELLER, M., SPANOU, Z., SCHAERLI, P., BRITSCHGI, M., YAWALKAR, N., SEITZ, M., VILLIGER, P. M. & PICHLER, W. J. 2005. T cell-regulated neutrophilic inflammation in autoinflammatory diseases. *J Immunol,* 175**,** 7678-86.

KHATRI, P., SIROTA, M. & BUTTE, A. J. 2012. Ten years of pathway analysis: current approaches and outstanding challenges. *PLoS Comput Biol,* 8**,** e1002375.

KHOJASTEH, S. C. 2011. *Drug metabolism and pharmacokinetics quick guide,* New York, Springer.

KILMARX, P. H. 2009. Global epidemiology of HIV. *Curr Opin HIV AIDS,* 4**,** 240-6.

KIM, S. H., CHOI, J. H., LEE, K. W., KIM, S. H., SHIN, E. S., OH, H. B., SUH, C. H., NAHM, D. H. & PARK, H. S. 2005. The human leucocyte antigen-DRB1*1302-DQB1*0609-DPB1*0201 haplotype may be a strong genetic marker for aspirin-induced urticaria. *Clin Exp Allergy,* 35**,** 339-44.

KIM, S. H., HUR, G. Y., CHOI, J. H. & PARK, H. S. 2008. Pharmacogenetics of aspirin-intolerant asthma. *Pharmacogenomics,* 9**,** 85-91.

KIM, S. H., LEE, K. W., SONG, W. J., KIM, S. H., JEE, Y. K., LEE, S. M., KANG, H. R., PARK, H. W., CHO, S. H., PARK, S. H.*, et al.* 2011. Carbamazepine-induced severe cutaneous adverse reactions and HLA genotypes in Koreans. *Epilepsy Res,* 97**,** 190-7.

KINDMARK, A., JAWAID, A., HARBRON, C. G., BARRATT, B. J., BENGTSSON, O. F., ANDERSSON, T. B., CARLSSON, S., CEDERBRANT, K. E., GIBSON, N. J., ARMSTRONG, M.*, et al.* 2008. Genome-wide pharmacogenetic investigation of a hepatic adverse event without clinical signs of immunopathology suggests an underlying immune pathogenesis. *Pharmacogenomics J,* 8**,** 186-95.

KISSEL, K., SANTOSO, S., HOFMANN, C., STRONCEK, D. & BUX, J. 2001. Molecular basis of the neutrophil glycoprotein NB1 (CD177) involved in the pathogenesis of immune neutropenias and transfusion reactions. *Eur J Immunol,* 31**,** 1301-9.

KISSEL, K., SCHEFFLER, S., KEROWGAN, M. & BUX, J. 2002. Molecular basis of NB1 (HNA-2a, CD177) deficiency. *Blood,* 99**,** 4231-3.

KLEIN, J. & SATO, A. 2000. The HLA system. First of two parts. *N Engl J Med,* 343**,** 702-9.

KLIPPEL, S., STRUNCK, E., BUSSE, C. E., BEHRINGER, D. & PAHL, H. L. 2002. Biochemical characterization of PRV-1, a novel hematopoietic cell surface receptor, which is overexpressed in polycythemia rubra vera. *Blood,* 100**,** 2441-8.

KNOBEL, H., GUELAR, A., MONTERO, M., CARMONA, A., LUQUE, S., BERENGUER, N. & GONZALEZ, A. 2008. Risk of side effects associated with the use of nevirapine in treatment-naive patients, with respect to gender and CD4 cell count. *HIV Med,* 9**,** 14-8.

KO, T. M., CHUNG, W. H., WEI, C. Y., SHIH, H. Y., CHEN, J. K., LIN, C. H., CHEN, Y. T. & HUNG, S. I. 2011. Shared and restricted T-cell receptor use is crucial for carbamazepine-induced Stevens-Johnson syndrome. *J Allergy Clin Immunol,* 128**,** 1266-1276 e11.

KOMURA, K., HASEGAWA, M., HAMAGUCHI, Y., YUKAMI, T., NAGAI, M., YACHIE, A., SATO, S. & TAKEHARA, K. 2005. Drug-induced hypersensitivity syndrome associated with human herpesvirus 6 and cytomegalovirus reactivation. *J Dermatol,* 32**,** 976-81.

KRALOVICS, R., PASSAMONTI, F., BUSER, A. S., TEO, S. S., TIEDT, R., PASSWEG, J. R., TICHELLI, A., CAZZOLA, M. & SKODA, R. C. 2005. A gain-of-function mutation of JAK2 in myeloproliferative disorders. *N Engl J Med,* 352**,** 1779-90.

KROH, E. M., PARKIN, R. K., MITCHELL, P. S. & TEWARI, M. 2010. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). *Methods,* 50**,** 298-301.

KROL, J., LOEDIGE, I. & FILIPOWICZ, W. 2010. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet,* 11**,** 597-610.

KULKARNI, S., SAVAN, R., QI, Y., GAO, X., YUKI, Y., BASS, S. E., MARTIN, M. P., HUNT, P., DEEKS, S. G., TELENTI, A.*, et al.* 2011. Differential microRNA regulation of HLA-C expression and its association with HIV control. *Nature,* 472**,** 495-8.

LALEZARI, P., MURPHY, G. B. & ALLEN, F. H., Jr. 1971. NB1, a new neutrophil-specific antigen involved in the pathogenesis of neonatal neutropenia. *J Clin Invest,* 50**,** 1108-15.

LAMORDE, M., BYAKIKA-KIBWIKA, P., OKABA-KAYOM, V., RYAN, M., COAKLEY, P., BOFFITO, M., NAMAKULA, R., KALEMEERA, F., COLEBUNDERS, R., BACK, D.*, et al.* 2011. Nevirapine pharmacokinetics when initiated at 200 mg or 400 mg daily in HIV-1 and tuberculosis co-infected Ugandan adults on rifampicin. *J Antimicrob Chemother,* 66**,** 180-3.

LANDSTEINER, K. & JACOBS, J. 1935. Studies on the Sensitization of Animals with Simple Chemical Compounds. *J Exp Med,* 61**,** 643-56.

LAWRIE, C. H., GAL, S., DUNLOP, H. M., PUSHKARAN, B., LIGGINS, A. P., PULFORD, K., BANHAM, A. H., PEZZELLA, F., BOULTWOOD, J., WAINSCOAT, J. S.*, et al.* 2008. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. *Br J Haematol,* 141**,** 672-5.

LAZAROU, J., POMERANZ, B. H. & COREY, P. N. 1998. Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. *JAMA,* 279**,** 1200-5.

LEE, W. M. 2003. Drug-induced hepatotoxicity. *N Engl J Med,* 349**,** 474-85.

LEVY, J. A. 1988. The transmission of AIDS: the case of the infected cell. *JAMA,* 259**,** 3037-8.

LEVY, J. A. 2009. HIV pathogenesis: 25 years of progress and persistent challenges. *AIDS,* 23**,** 147-60.

LI, J. & UETRECHT, J. P. 2010. The danger hypothesis applied to idiosyncratic drug reactions. *Handb Exp Pharmacol***,** 493-509.

LIKANONSAKUL, S., RATTANATHAM, T., FEANGVAD, S., UTTAYAMAKUL, S., PRASITHSIRIKUL, W., TUNTHANATHIP, P., NAKAYAMA, E. E. & SHIODA, T. 2009. HLA-Cw*04 allele associated with nevirapine-induced rash in HIV-infected Thai patients. *AIDS Res Ther,* 6**,** 22.

LILL, M., KOKS, S., SOOMETS, U., SCHALKWYK, L. C., FERNANDES, C., LUTSAR, I. & TABA, P. 2013. Peripheral blood RNA gene expression profiling in patients with bacterial meningitis. *Front Neurosci,* 7**,** 33.

LINK, E., PARISH, S., ARMITAGE, J., BOWMAN, L., HEATH, S., MATSUDA, F., GUT, I., LATHROP, M., COLLINS, R. & SEARCH COLLABORATIVE GROUP 2008. SLCO1B1 variants and statin-induced myopathy--a genomewide study. *N Engl J Med,* 359**,** 789-99.

LIPTROTT, N. J., PUSHPAKOM, S., WYEN, C., FATKENHEUER, G., HOFFMANN, C., MAUSS, S., KNECHTEN, H., BROCKMEYER, N. H., HOPPER-BORGE, E., SICCARDI, M.*, et al.* 2012. Association of ABCC10 polymorphisms with nevirapine plasma concentrations in the German Competence Network for HIV/AIDS. *Pharmacogenet Genomics,* 22**,** 10-9.

LITTERA, R., CARCASSI, C., MASALA, A., PIANO, P., SERRA, P., ORTU, F., CORSO, N., CASULA, B., LA NASA, G., CONTU, L.*, et al.* 2006. HLA-dependent hypersensitivity to nevirapine in Sardinian HIV patients. *AIDS,* 20**,** 1621-6.

LIU, E., JELINEK, J., PASTORE, Y. D., GUAN, Y., PRCHAL, J. F. & PRCHAL, J. T. 2003. Discrimination of polycythemias and thrombocytoses by novel, simple, accurate clonality assays and comparison with PRV-1 expression and BFU-E response to erythropoietin. *Blood,* 101**,** 3294-301.

LIU, X., ZHAN, Z., XU, L., MA, F., LI, D., GUO, Z., LI, N. & CAO, X. 2010. MicroRNA-148/152 impair innate response and antigen presentation of TLR-triggered dendritic cells by targeting CaMKIIalpha. *J Immunol,* 185**,** 7244-51.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25**,** 402-8.

LONJOU, C., BOROT, N., SEKULA, P., LEDGER, N., THOMAS, L., HALEVY, S., NALDI, L., BOUWES-BAVINCK, J. N., SIDOROFF, A., DE TOMA, C.*, et al.* 2008. A European study of HLA-B in Stevens-Johnson syndrome and toxic epidermal necrolysis related to five high-risk drugs. *Pharmacogenet Genomics,* 18**,** 99-107.

LOTZE, M. T. & TRACEY, K. J. 2005. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol,* 5**,** 331-42.

LUCENA, M. I., MOLOKHIA, M., SHEN, Y., URBAN, T. J., AITHAL, G. P., ANDRADE, R. J., DAY, C. P., RUIZ-CABELLO, F., DONALDSON, P. T., STEPHENS, C.*, et al.* 2011. Susceptibility to amoxicillin-clavulanate-induced liver injury is influenced by multiple HLA class I and II alleles. *Gastroenterology,* 141**,** 338-47.

LYONS, F., HOPKINS, S., KELLEHER, B., MCGEARY, A., SHEEHAN, G., GEOGHEGAN, J., BERGIN, C., MULCAHY, F. M. & MCCORMICK, P. A. 2006. Maternal hepatotoxicity with nevirapine as part of combination antiretroviral therapy in pregnancy. *HIV Med,* 7**,** 255-60.

MA, Q. & LU, A. Y. 2011. Pharmacogenetics, pharmacogenomics, and individualized medicine. *Pharmacol Rev,* 63**,** 437-59.

MAHASIRIMONGKOL, S. 2013. Genotype Based Personalized Prescription of Nevirapine (GENPART). *National Library of Medicine.* Bethesda (MD), US: National Institute of Health.

MAHUNGU, T., SMITH, C., TURNER, F., EGAN, D., YOULE, M., JOHNSON, M., KHOO, S., BACK, D. & OWEN, A. 2009. Cytochrome P450 2B6 516G-->T is associated with plasma concentrations of nevirapine at both 200 mg twice daily and 400 mg once daily in an ethnically diverse population. *HIV Med,* 10**,** 310-7.

MALLAL, S., NOLAN, D., WITT, C., MASEL, G., MARTIN, A. M., MOORE, C., SAYER, D., CASTLEY, A., MAMOTTE, C., MAXWELL, D.*, et al.* 2002. Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. *Lancet,* 359**,** 727-32.

MALLAL, S., PHILLIPS, E., CAROSI, G., MOLINA, J. M., WORKMAN, C., TOMAZIC, J., JAGEL-GUEDES, E., RUGINA, S., KOZYREV, O., CID, J. F.*, et al.* 2008. HLA-B*5701 screening for hypersensitivity to abacavir. *N Engl J Med,* 358**,** 568-79.

MAN, C. B., KWAN, P., BAUM, L., YU, E., LAU, K. M., CHENG, A. S. & NG, M. H. 2007. Association between HLA-B*1502 allele and antiepileptic drug-induced cutaneous reactions in Han Chinese. *Epilepsia,* 48**,** 1015-8.

MANASTER, I., GOLDMAN-WOHL, D., GREENFIELD, C., NACHMANI, D., TSUKERMAN, P., HAMANI, Y., YAGEL, S. & MANDELBOIM, O. 2012. MiRNA-mediated control of HLA-G expression and function. *PLoS One,* 7**,** e33395.

MARSH, S. G., ALBERT, E. D., BODMER, W. F., BONTROP, R. E., DUPONT, B., ERLICH, H. A., FERNANDEZ-VINA, M., GERAGHTY, D. E., HOLDSWORTH, R., HURLEY, C. K.*, et al.* 2010. Nomenclature for factors of the HLA system, 2010. *Tissue Antigens,* 75**,** 291-455.

MARTIN, A. M., NOLAN, D., JAMES, I., CAMERON, P., KELLER, J., MOORE, C., PHILLIPS, E., CHRISTIANSEN, F. T. & MALLAL, S. 2005. Predisposition to nevirapine hypersensitivity associated with HLA-DRB1*0101 and abrogated by low CD4 T-cell counts. *AIDS,* 19**,** 97-9.

MATSUO, K., LIN, A., PROCTER, J. L., CLEMENT, L. & STRONCEK, D. 2000. Variations in the expression of granulocyte antigen NB1. *Transfusion,* 40**,** 654-62.

MATZINGER, P. 1994. Tolerance, danger, and the extended family. *Annu Rev Immunol,* 12**,** 991-1045.

MAX, B. & SHERER, R. 2000. Management of the adverse effects of antiretroviral therapy and medication adherence. *Clin Infect Dis,* 30 Suppl 2**,** S96-116.

MCCORMACK, M., ALFIREVIC, A., BOURGEOIS, S., FARRELL, J. J., KASPERAVICIUTE, D., CARRINGTON, M., SILLS, G. J., MARSON, T., JIA, X., DE BAKKER, P. I.*, et al.* 2011. HLA-A*3101 and carbamazepine-induced hypersensitivity reactions in Europeans. *N Engl J Med,* 364**,** 1134-43.

MCDONALD, J. S., MILOSEVIC, D., REDDI, H. V., GREBE, S. K. & ALGECIRAS-SCHIMNICH, A. 2011. Analysis of circulating microRNA: preanalytical and analytical challenges. *Clin Chem,* 57**,** 833-40.

MCKENNA, D. J., MCDADE, S. S., PATEL, D. & MCCANCE, D. J. 2010. MicroRNA 203 expression in keratinocytes is dependent on regulation of p53 levels by E6. *J Virol,* 84**,** 10644-52.

MEGA, J. L., SIMON, T., COLLET, J. P., ANDERSON, J. L., ANTMAN, E. M., BLIDEN, K., CANNON, C. P., DANCHIN, N., GIUSTI, B., GURBEL, P.*, et al.* 2010. Reduced-function CYP2C19 genotype and risk of adverse clinical outcomes among patients treated with clopidogrel predominantly for PCI: a meta-analysis. *JAMA,* 304**,** 1821-30.

MEHTA, T. Y., PRAJAPATI, L. M., MITTAL, B., JOSHI, C. G., SHETH, J. J., PATEL, D. B., DAVE, D. M. & GOYAL, R. K. 2009. Association of HLA-B*1502 allele and carbamazepine-induced Stevens-Johnson syndrome among Indians. *Indian J Dermatol Venereol Leprol,* 75**,** 579-82.

METRY, D. W., LAHART, C. J., FARMER, K. L. & HEBERT, A. A. 2001. Stevens-Johnson syndrome caused by the antiretroviral drug nevirapine. *J Am Acad Dermatol,* 44**,** 354-7.

MEYER, L., MAGIEROWSKA, M., HUBERT, J. B., ROUZIOUX, C., DEVEAU, C., SANSON, F., DEBRE, P., DELFRAISSY, J. F. & THEODOROU, I. 1997. Early protective effect of CCR-5 delta 32 heterozygosity on HIV-1 disease progression: relationship with viral load. The SEROCO Study Group. *AIDS,* 11**,** F73-8.

MEYER, U. A. 2000. Pharmacogenetics and adverse drug reactions. *Lancet,* 356**,** 1667-71.

MEYERSON, H. J., OSEI, E., SCHWEITZER, K., BLIDARU, G., EDINGER, A. & BALOG, A. 2013. CD177 expression on neutrophils: in search of a clonal assay for myeloid neoplasia by flow cytometry. *Am J Clin Pathol,* 140**,** 658-69.

MINERS, J. O., KNIGHTS, K. M., HOUSTON, J. B. & MACKENZIE, P. I. 2006. In vitro-in vivo correlation for drugs and other compounds eliminated by glucuronidation in humans: pitfalls and promises. *Biochem Pharmacol,* 71**,** 1531-9.

MITCHELL, P. S., PARKIN, R. K., KROH, E. M., FRITZ, B. R., WYMAN, S. K., POGOSOVA-AGADJANYAN, E. L., PETERSON, A., NOTEBOOM, J., O'BRIANT, K. C., ALLEN, A.*, et al.* 2008. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A,* 105**,** 10513-8.

MITTMANN, N., KNOWLES, S. R., KOO, M., SHEAR, N. H., RACHLIS, A. & ROURKE, S. B. 2012. Incidence of toxic epidermal necrolysis and Stevens-Johnson Syndrome in an

HIV cohort: an observational, retrospective case series study. *Am J Clin Dermatol,* 13**,** 49-54.

MONTASER, L. M., EL-RASHIDI, F. H., ESSA, E. S. & AZAB, S. M. 2011. Analysis of CD177 neutrophil expression in beta-thalassemia patients. *APMIS,* 119**,** 674-80.

MORITZ, E., CHIBA, A. K., KIMURA, E. Y., ALBUQUERQUE, D., GUIRAO, F. P., YAMAMOTO, M., COSTA, F. F. & BORDIN, J. O. 2010. Molecular studies reveal that A134T, G156A and G1333A SNPs in the CD177 gene are associated with atypical expression of human neutrophil antigen-2. *Vox Sang,* 98**,** 160-6.

MUNZ, C. 2012. Antigen Processing for MHC Class II Presentation via Autophagy. *Front Immunol,* 3**,** 9.

MURAKAMI, T. & YAMAMOTO, N. 2010. Role of CXCR4 in HIV infection and its potential as a therapeutic target. *Future Microbiol,* 5**,** 1025-39.

MURPHY, K., TRAVERS, P., WALPORT, M. & JANEWAY, C. 2012. *Janeway's immunobiology,* New York, Garland Science.

NAKASA, T., MIYAKI, S., OKUBO, A., HASHIMOTO, M., NISHIDA, K., OCHI, M. & ASAHARA, H. 2008. Expression of microRNA-146 in rheumatoid arthritis synovial tissue. *Arthritis Rheum,* 58**,** 1284-92.

NARANJO, C. A., BUSTO, U., SELLERS, E. M., SANDOR, P., RUIZ, I., ROBERTS, E. A., JANECEK, E., DOMECQ, C. & GREENBLATT, D. J. 1981. A method for estimating the probability of adverse drug reactions. *Clin Pharmacol Ther,* 30**,** 239-45.

NASSIF, A., BENSUSSAN, A., BOUMSELL, L., DENIAUD, A., MOSLEHI, H., WOLKENSTEIN, P., BAGOT, M. & ROUJEAU, J. C. 2004. Toxic epidermal necrolysis: effector cells are drug-specific cytotoxic T cells. *J Allergy Clin Immunol,* 114**,** 1209-15.

NASSIF, A., BENSUSSAN, A., DOROTHEE, G., MAMI-CHOUAIB, F., BACHOT, N., BAGOT, M., BOUMSELL, L. & ROUJEAU, J. C. 2002. Drug specific cytotoxic T-cells in the skin lesions of a patient with toxic epidermal necrolysis. *J Invest Dermatol,* 118**,** 728-33.

NEBERT, D. W., ADESNIK, M., COON, M. J., ESTABROOK, R. W., GONZALEZ, F. J., GUENGERICH, F. P., GUNSALUS, I. C., JOHNSON, E. F., KEMPER, B., LEVIN, W.*, et al.* 1987. The P450 gene superfamily: recommended nomenclature. *DNA,* 6**,** 1-11.

NEUHAUS, J., JACOBS, D. R., Jr., BAKER, J. V., CALMY, A., DUPREZ, D., LA ROSA, A., KULLER, L. H., PETT, S. L., RISTOLA, M., ROSS, M. J.*, et al.* 2010. Markers of inflammation, coagulation, and renal function are elevated in adults with HIV infection. *J Infect Dis,* 201**,** 1788-95.

NG, P. C. & HENIKOFF, S. 2001. Predicting deleterious amino acid substitutions. *Genome Res,* 11**,** 863-74.

NOBLE, W. S. 2009. How does multiple testing correction work? *Nat Biotech,* 27**,** 1135-1137.

NOMURA, L., MAINO, V. C. & MAECKER, H. T. 2008. Standardization and optimization of multiparameter intracellular cytokine staining. *Cytometry A,* 73**,** 984-91.

NORCROSS, M. A., LUO, S., LU, L., BOYNE, M. T., GOMARTELI, M., RENNELS, A. D., WOODCOCK, J., MARGULIES, D. H., MCMURTREY, C., VERNON, S.*, et al.* 2012. Abacavir induces loading of novel self-peptides into HLA-B*57: 01: an autoimmune model for HLA-associated drug hypersensitivity. *AIDS,* 26**,** F21-9.

NYFELER, B. & PICHLER, W. J. 1997. The lymphocyte transformation test for the diagnosis of drug allergy: sensitivity and specificity. *Clin Exp Allergy,* 27**,** 175-81.

O'BRIEN, S. J. & MOORE, J. P. 2000. The effect of genetic variation in chemokines and their receptors on HIV transmission and progression to AIDS. *Immunol Rev,* 177**,** 99-111.

O'BRIEN, T. R., WINKLER, C., DEAN, M., NELSON, J. A., CARRINGTON, M., MICHAEL, N. L. & WHITE, G. C., 2nd 1997. HIV-1 infection in a man homozygous for CCR5 delta 32. *Lancet,* 349**,** 1219.

O'HUIGIN, C., KULKARNI, S., XU, Y., DENG, Z., KIDD, J., KIDD, K., GAO, X. & CARRINGTON, M. 2011. The molecular origin and consequences of escape from miRNA regulation by HLA-C alleles. *Am J Hum Genet,* 89**,** 424-31.

OCHS, M. J., STEINHILBER, D. & SUESS, B. 2011. MicroRNA Involved in Inflammation: Control of Eicosanoid Pathway. *Front Pharmacol,* 2**,** 39.

OJINGWA, J. C., SPAHN-LANGGUTH, H. & BENET, L. Z. 1994. Reversible binding of tolmetin, zomepirac, and their glucuronide conjugates to human serum albumin and plasma. *J Pharmacokinet Biopharm,* 22**,** 19-40.

OSTROV, D. A., GRANT, B. J., POMPEU, Y. A., SIDNEY, J., HARNDAHL, M., SOUTHWOOD, S., OSEROFF, C., LU, S., JAKONCIC, J., DE OLIVEIRA, C. A.*, et al.* 2012. Drug hypersensitivity caused by alteration of the MHC-presented self-peptide repertoire. *Proc Natl Acad Sci U S A,* 109**,** 9959-64.

OZEKI, T., MUSHIRODA, T., YOWANG, A., TAKAHASHI, A., KUBO, M., SHIRAKATA, Y., IKEZAWA, Z., IIJIMA, M., SHIOHARA, T., HASHIMOTO, K.*, et al.* 2011. Genome-wide association study identifies HLA-A*3101 allele as a genetic risk factor for carbamazepine-induced cutaneous adverse drug reactions in Japanese population. *Hum Mol Genet,* 20**,** 1034-41.

OZKAYA-BAYAZIT, E. & AKAR, U. 2001. Fixed drug eruption induced by trimethoprim-sulfamethoxazole: evidence for a link to HLA-A30 B13 Cw6 haplotype. *J Am Acad Dermatol,* 45**,** 712-7.

PAN, W., ZHU, S., YUAN, M., CUI, H., WANG, L., LUO, X., LI, J., ZHOU, H., TANG, Y. & SHEN, N. 2010. MicroRNA-21 and microRNA-148a contribute to DNA hypomethylation in lupus CD4+ T cells by directly and indirectly targeting DNA methyltransferase 1. *J Immunol,* 184**,** 6773-81.

PAQUET, P. & PIERARD, G. E. 2002. Differential pathomechanisms of epidermal necrolytic blistering diseases. *Int J Mol Med,* 10**,** 695-9.

PARK, B. K., LAVERTY, H., SRIVASTAVA, A., ANTOINE, D. J., NAISBITT, D. & WILLIAMS, D. P. 2011. Drug bioactivation and protein adduct formation in the pathogenesis of drug-induced toxicity. *Chem Biol Interact,* 192**,** 30-6.

PARK, B. K., PIRMOHAMED, M. & KITTERINGHAM, N. R. 1995. The role of cytochrome P450 enzymes in hepatic and extrahepatic human drug toxicity. *Pharmacol Ther,* 68**,** 385-424.

PATEL, S. M., JOHNSON, S., BELKNAP, S. M., CHAN, J., SHA, B. E. & BENNETT, C. 2004. Serious adverse cutaneous and hepatic toxicities associated with nevirapine use by non-HIV-infected individuals. *J Acquir Immune Defic Syndr,* 35**,** 120-5.

PAULEY, K. M., SATOH, M., CHAN, A. L., BUBB, M. R., REEVES, W. H. & CHAN, E. K. 2008. Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients. *Arthritis Res Ther,* 10**,** R101.

PELLICANO, R., LOMUTO, M., CIAVARELLA, G., DI GIORGIO, G. & GASPARINI, P. 1997. Fixed drug eruptions with feprazone are linked to HLA-B22. *J Am Acad Dermatol,* 36**,** 782-4.

PENZAK, S. R., KABUYE, G., MUGYENYI, P., MBAMANYA, F., NATARAJAN, V., ALFARO, R. M., KITYO, C., FORMENTINI, E. & MASUR, H. 2007. Cytochrome P450 2B6 (CYP2B6) G516T influences nevirapine plasma concentrations in HIV-infected patients in Uganda. *HIV Med,* 8**,** 86-91.

PEREIRA, F. A., MUDGIL, A. V. & ROSMARIN, D. M. 2007. Toxic epidermal necrolysis. *J Am Acad Dermatol,* 56**,** 181-200.

PETERS, P. J., STRINGER, J., MCCONNELL, M. S., KIARIE, J., RATANASUWAN, W., INTALAPAPORN, P., POTTER, D., MUTSOTSO, W., ZULU, I., BORKOWF, C. B.*, et al.* 2010. Nevirapine-associated hepatotoxicity was not predicted by CD4 count >/=250 cells/muL among women in Zambia, Thailand and Kenya. *HIV Med,* 11**,** 650-60.

PHILLIPS, E., BARTLETT, J. A., SANNE, I., LEDERMAN, M. M., HINKLE, J., ROUSSEAU, F., DUNN, D., PAVLOS, R., JAMES, I., MALLAL, S. A.*, et al.* 2013. Associations between HLA-DRB1*0102, HLA-B*5801, and hepatotoxicity during initiation of nevirapine-containing regimens in South Africa. *J Acquir Immune Defic Syndr,* 62**,** e55-7.

PHILLIPS, E., KEANE, N., BLYTH, C., LUCAS, A., MCKINNON, E. & MALLAL, S. Both HLA Class I Restricted CD8+ and Class II Restricted CD4+T Cells are Implicated in the Pathogenesis of Nevirapine Hypersensitivity. 51st ICAAC, 17-20 November 2011 Chicago, IL, USA.

PHILLIPS, E. J. & MALLAL, S. A. 2010. Pharmacogenetics of drug hypersensitivity. *Pharmacogenomics,* 11**,** 973-87.

PHILLIPS, E. J., SULLIVAN, J. R., KNOWLES, S. R. & SHEAR, N. H. 2002. Utility of patch testing in patients with hypersensitivity syndromes associated with abacavir. *AIDS,* 16**,** 2223-5.

PICARD, D., JANELA, B., DESCAMPS, V., D'INCAN, M., COURVILLE, P., JACQUOT, S., ROGEZ, S., MARDIVIRIN, L., MOINS-TEISSERENC, H., TOUBERT, A.*, et al.* 2010. Drug reaction with eosinophilia and systemic symptoms (DRESS): a multiorgan antiviral T cell response. *Sci Transl Med,* 2**,** 46ra62.

PICHLER, W. J. 2002. Pharmacological interaction of drugs with antigen-specific immune receptors: the p-i concept. *Curr Opin Allergy Clin Immunol,* 2**,** 301-5.

PICHLER, W. J. 2003. Delayed drug hypersensitivity reactions. *Ann Intern Med,* 139**,** 683-93.

PICHLER, W. J., ADAM, J., DAUBNER, B., GENTINETTA, T., KELLER, M. & YERLY, D. 2010. Drug hypersensitivity reactions: pathomechanism and clinical symptoms. *Med Clin North Am,* 94**,** 645-64, xv.

PICHLER, W. J., BEELER, A., KELLER, M., LERCH, M., POSADAS, S., SCHMID, D., SPANOU, Z., ZAWODNIAK, A. & GERBER, B. 2006. Pharmacological interaction of drugs with immune receptors: the p-i concept. *Allergol Int,* 55**,** 17-25.

PICHLER, W. J., YAWALKAR, N., BRITSCHGI, M., DEPTA, J., STRASSER, I., SCHMID, S., KUECHLER, P. & NAISBITT, D. 2002. Cellular and molecular pathophysiology of cutaneous drug reactions. *Am J Clin Dermatol,* 3**,** 229-38.

PIRMOHAMED, M. 2007. HIV and drug hypersensitivity. *In:* PICHLER, W. J. (ed.) *Drug hypersensitivity.* Basel ; New York: Karger.

PIRMOHAMED, M. 2010. Pharmacogenetics of idiosyncratic adverse drug reactions. *Handb Exp Pharmacol***,** 477-91.

PIRMOHAMED, M., BRECKENRIDGE, A. M., KITTERINGHAM, N. R. & PARK, B. K. 1998. Adverse drug reactions. *BMJ,* 316**,** 1295-8.

PIRMOHAMED, M., FRIEDMANN, P. S., MOLOKHIA, M., LOKE, Y. K., SMITH, C., PHILLIPS, E., LA GRENADE, L., CARLETON, B., PAPALUCA-AMATI, M., DEMOLY, P.*, et al.* 2011. Phenotype standardization for immune-mediated drug-induced skin injury. *Clin Pharmacol Ther,* 89**,** 896-901.

PIRMOHAMED, M., JAMES, S., MEAKIN, S., GREEN, C., SCOTT, A. K., WALLEY, T. J., FARRAR, K., PARK, B. K. & BRECKENRIDGE, A. M. 2004. Adverse drug reactions as cause of admission to hospital: prospective analysis of 18 820 patients. *BMJ,* 329**,** 15-9.

PIRMOHAMED, M., NAISBITT, D. J., GORDON, F. & PARK, B. K. 2002. The danger hypothesis--potential role in idiosyncratic drug reactions. *Toxicology,* 181-182**,** 55-63.

POLLARD, R. B., ROBINSON, P. & DRANSFIELD, K. 1998. Safety profile of nevirapine, a nonnucleoside reverse transcriptase inhibitor for the treatment of human immunodeficiency virus infection. *Clin Ther,* 20**,** 1071-92.

PRITCHARD, C. C., CHENG, H. H. & TEWARI, M. 2012. MicroRNA profiling: approaches and considerations. *Nat Rev Genet,* 13**,** 358-69.

QIAGEN. *miScript miRNA PCR Arrays, Functional Gene Grouping,* [Online]. Available: http://www.qiagen.com/products/catalog/assay-technologies/mirna/miscript-mirna-pcr-arrays?catno=MIHS-106Z#geneglobe [Accessed January 2014].

QUIRALTE, J., SANCHEZ-GARCIA, F., TORRES, M. J., BLANCO, C., CASTILLO, R., ORTEGA, N., DE CASTRO, F. R., PEREZ-ACIEGO, P. & CARRILLO, T. 1999. Association of HLA-DR11 with the anaphylactoid reaction caused by nonsteroidal anti-inflammatory drugs. *J Allergy Clin Immunol,* 103**,** 685-9.

R CORE TEAM. 2013. *R: A Language and Environment for Statistical Computing* [Online]. Vienna, Austria: R Foundation for Statistical Computing. Available: http://www.R-project.org [2013].

RABINOWITS, G., GERCEL-TAYLOR, C., DAY, J. M., TAYLOR, D. D. & KLOECKER, G. H. 2009. Exosomal microRNA: a diagnostic marker for lung cancer. *Clin Lung Cancer,* 10**,** 42-6.

RAMACHANDRAN, G., RAMESH, K., HEMANTH KUMAR, A. K., JAGAN, I., VASANTHA, M., PADMAPRIYADARSINI, C., NARENDRAN, G., RAJASEKARAN, S. & SWAMINATHAN, S. 2009. Association of high T allele frequency of CYP2B6 G516T polymorphism among ethnic south Indian HIV-infected patients with elevated plasma efavirenz and nevirapine. *J Antimicrob Chemother,* 63**,** 841-3.

RAMIREZ-VELAZQUEZ, C., CASTILLO, E. C., GUIDO-BAYARDO, L. & ORTIZ-NAVARRETE, V. 2013. IL-17-producing peripheral blood CD177+ neutrophils increase in allergic asthmatic subjects. *Allergy Asthma Clin Immunol,* 9**,** 23.

RAUCH, A., NOLAN, D., MARTIN, A., MCKINNON, E., ALMEIDA, C. & MALLAL, S. 2006. Prospective genetic screening decreases the incidence of abacavir hypersensitivity reactions in the Western Australian HIV cohort study. *Clin Infect Dis,* 43**,** 99-102.

RAWLINGS, M. D. & THOMPSON, J. W. 1977. Pathogenesis of adverse drug reactions. *In:* DAVIES, D. M. (ed.) *Textbook of adverse drug reactions.* Oxford ; New York: Oxford University Press.

REEVES, J. D. & DOMS, R. W. 2002. Human immunodeficiency virus type 2. *J Gen Virol,* 83**,** 1253-65.

RESNICK, K. E., ALDER, H., HAGAN, J. P., RICHARDSON, D. L., CROCE, C. M. & COHN, D. E. 2009. The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform. *Gynecol Oncol,* 112**,** 55-9.

RICHMAN, D. D., HAVLIR, D., CORBEIL, J., LOONEY, D., IGNACIO, C., SPECTOR, S. A., SULLIVAN, J., CHEESEMAN, S., BARRINGER, K., PAULETTI, D.*, et al.* 1994. Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. *J Virol,* 68**,** 1660-6.

RISKA, P., LAMSON, M., MACGREGOR, T., SABO, J., HATTOX, S., PAV, J. & KEIRNS, J. 1999a. Disposition and biotransformation of the antiretroviral drug nevirapine in humans. *Drug Metab Dispos,* 27**,** 895-901.

RISKA, P. S., JOSEPH, D. P., DINALLO, R. M., DAVIDSON, W. C., KEIRNS, J. J. & HATTOX, S. E. 1999b. Biotransformation of nevirapine, a non-nucleoside HIV-1 reverse transcriptase inhibitor, in mice, rats, rabbits, dogs, monkeys, and chimpanzees. *Drug Metab Dispos,* 27**,** 1434-47.

RITCHIE, M. D., HAAS, D. W., MOTSINGER, A. A., DONAHUE, J. P., ERDEM, H., RAFFANTI, S., REBEIRO, P., GEORGE, A. L., KIM, R. B., HAINES, J. L.*, et al.* 2006. Drug transporter and metabolizing enzyme gene variants and nonnucleoside reverse-transcriptase inhibitor hepatotoxicity. *Clin Infect Dis,* 43**,** 779-82.

ROBINSON, J., MISTRY, K., MCWILLIAM, H., LOPEZ, R., PARHAM, P. & MARSH, S. G. 2011. The IMGT/HLA database. *Nucleic Acids Res,* 39**,** D1171-6.

ROSS, C. J., KATZOV-ECKERT, H., DUBE, M. P., BROOKS, B., RASSEKH, S. R., BARHDADI, A., FEROZ-ZADA, Y., VISSCHER, H., BROWN, A. M., RIEDER, M. J.*, et al.* 2009. Genetic variants in TPMT and COMT are associated with hearing loss in children receiving cisplatin chemotherapy. *Nat Genet,* 41**,** 1345-9.

ROTGER, M., COLOMBO, S., FURRER, H., BLEIBER, G., BUCLIN, T., LEE, B. L., KEISER, O., BIOLLAZ, J., DECOSTERD, L., TELENTI, A.*, et al.* 2005. Influence of CYP2B6 polymorphism on plasma and intracellular concentrations and toxicity of efavirenz and nevirapine in HIV-infected patients. *Pharmacogenet Genomics,* 15**,** 1-5.

ROUJEAU, J. C. 2005. Clinical heterogeneity of drug hypersensitivity. *Toxicology,* 209**,** 123-9.

ROUJEAU, J. C., HUYNH, T. N., BRACQ, C., GUILLAUME, J. C., REVUZ, J. & TOURAINE, R. 1987. Genetic susceptibility to toxic epidermal necrolysis. *Arch Dermatol,* 123**,** 1171-3.

RZANY, B., MOCKENHAUPT, M., STOCKER, U., HAMOUDA, O. & SCHOPF, E. 1993. Incidence of Stevens-Johnson syndrome and toxic epidermal necrolysis in patients with the acquired immunodeficiency syndrome in Germany. *Arch Dermatol,* 129**,** 1059.

SABIOSCIENCES. *miScript miRNA PCR Array Data Analysis* [Online]. QIAGEN. Available: http://pcrdataanalysis.sabiosciences.com/mirna/arrayanalysis.php [Accessed January 2014].

SACHS, U. J., ANDREI-SELMER, C. L., MANIAR, A., WEISS, T., PADDOCK, C., ORLOVA, V. V., CHOI, E. Y., NEWMAN, P. J., PREISSNER, K. T., CHAVAKIS, T.*, et al.* 2007. The neutrophil-specific antigen CD177 is a counter-receptor for platelet endothelial cell adhesion molecule-1 (CD31). *J Biol Chem,* 282**,** 23603-12.

SAEZ-CIRION, A., PANCINO, G., SINET, M., VENET, A., LAMBOTTE, O. & GROUP, A. E. H. C. S. 2007. HIV controllers: how do they tame the virus? *Trends Immunol,* 28**,** 532-40.

SANCHEZ-MAZAS, A., DJOULAH, S., BUSSON, M., LE MONNIER DE GOUVILLE, I., POIRIER, J. C., DEHAY, C., CHARRON, D., EXCOFFIER, L., SCHNEIDER, S., LANGANEY, A.*, et al.* 2000. A linkage disequilibrium map of the MHC region based on the analysis of 14 loci haplotypes in 50 French families. *Eur J Hum Genet,* 8**,** 33-41.

SANDERSON, J. P., NAISBITT, D. J. & PARK, B. K. 2006. Role of bioactivation in drug-induced hypersensitivity reactions. *AAPS J,* 8**,** E55-64.

SATTENTAU, Q. J. & MOORE, J. P. 1993. The role of CD4 in HIV binding and entry. *Philos Trans R Soc Lond B Biol Sci,* 342**,** 59-66.

SCHAERLI, P., BRITSCHGI, M., KELLER, M., STEINER, U. C., STEINMANN, L. S., MOSER, B. & PICHLER, W. J. 2004. Characterization of human T cells that regulate neutrophilic skin inflammation. *J Immunol,* 173**,** 2151-8.

SEISHIMA, M., YAMANAKA, S., FUJISAWA, T., TOHYAMA, M. & HASHIMOTO, K. 2006. Reactivation of human herpesvirus (HHV) family members other than HHV-6 in drug-induced hypersensitivity syndrome. *Br J Dermatol,* 155**,** 344-9.

SETTE, A. & SIDNEY, J. 1998. HLA supertypes and supermotifs: a functional perspective on HLA polymorphism. *Curr Opin Immunol,* 10**,** 478-82.

SHAFFER, J., SCHLUMPBERGER, M. & LADER, E. 2012. miRNA profiling from blood - challenges and recommendations. *SA Biosciences - Manuals* [Online]. Available: http://www.sabiosciences.com/manuals/Whitepaper_Serumplasma.pdf.

SHAHABI, V., BERMAN, D., CHASALOW, S. D., WANG, L., TSUCHIHASHI, Z., HU, B., PANTING, L., JURE-KUNKEL, M. & JI, R. R. 2013. Gene expression profiling of whole blood in ipilimumab-treated patients for identification of potential biomarkers of immune-related gastrointestinal adverse events. *J Transl Med,* 11**,** 75.

SHARKEY, J. W., ANTOINE, D. J. & PARK, B. K. 2012. Validation of the isolation and quantification of kidney enriched miRNAs for use as biomarkers. *Biomarkers,* 17**,** 231-9.

SHARMA, A. M., KLARSKOV, K. & UETRECHT, J. 2013a. Nevirapine bioactivation and covalent binding in the skin. *Chem Res Toxicol,* 26**,** 410-21.

SHARMA, A. M., LI, Y., NOVALEN, M., HAYES, M. A. & UETRECHT, J. 2012. Bioactivation of nevirapine to a reactive quinone methide: implications for liver injury. *Chem Res Toxicol,* 25**,** 1708-19.

SHARMA, A. M., NOVALEN, M., TANINO, T. & UETRECHT, J. P. 2013b. 12-OH-nevirapine sulfate, formed in the skin, is responsible for nevirapine-induced skin rash. *Chem Res Toxicol,* 26**,** 817-27.

SHENTON, J. M., POPOVIC, M., CHEN, J., MASSON, M. J. & UETRECHT, J. P. 2005. Evidence of an immune-mediated mechanism for an idiosyncratic nevirapine-induced reaction in the female Brown Norway rat. *Chem Res Toxicol,* 18**,** 1799-813.

SHENTON, J. M., TERANISHI, M., ABU-ASAB, M. S., YAGER, J. A. & UETRECHT, J. P. 2003. Characterization of a potential animal model of an idiosyncratic drug reaction: nevirapine-induced skin rash in the rat. *Chem Res Toxicol,* 16**,** 1078-89.

SHEPPARD, H. W., CELUM, C., MICHAEL, N. L., O'BRIEN, S., DEAN, M., CARRINGTON, M., DONDERO, D. & BUCHBINDER, S. P. 2002. HIV-1 infection in individuals with the CCR5-Delta32/Delta32 genotype: acquisition of syncytium-inducing virus at seroconversion. *J Acquir Immune Defic Syndr,* 29**,** 307-13.

SHERRY, S. T., WARD, M. H., KHOLODOV, M., BAKER, J., PHAN, L., SMIGIELSKI, E. M. & SIROTKIN, K. 2001. dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res,* 29**,** 308-11.

SHIFENG, H., DANNI, W., PU, C., PING, Y., JU, C. & LIPING, Z. 2013. Circulating Liver-Specific miR-122 as a Novel Potential Biomarker for Diagnosis of Cholestatic Liver Injury. *PLoS ONE,* 8**,** e73133.

SHUBBER, Z., CALMY, A., ANDRIEUX-MEYER, I., VITORIA, M., RENAUD-THERY, F., SHAFFER, N., HARGREAVES, S., MILLS, E. J. & FORD, N. 2013. Adverse events associated with nevirapine and efavirenz-based first-line antiretroviral therapy: a systematic review and meta-analysis. *AIDS,* 27**,** 1403-12.

SIERRA, S., KUPFER, B. & KAISER, R. 2005. Basics of the virology of HIV-1 and its replication. *J Clin Virol,* 34**,** 233-44.

SINGER, J. B., LEWITZKY, S., LEROY, E., YANG, F., ZHAO, X., KLICKSTEIN, L., WRIGHT, T. M., MEYER, J. & PAULDING, C. A. 2010. A genome-wide study identifies HLA alleles associated with lumiracoxib-related liver injury. *Nat Genet,* 42**,** 711-4.

SIRHAN, S., LASHO, T. L., ELLIOTT, M. A. & TEFFERI, A. 2005. Neutrophil polycythemia rubra vera-1 expression in classic and atypical myeloproliferative disorders and laboratory correlates. *Haematologica,* 90**,** 406-8.

SLEZAK, S., JIN, P., CARUCCIO, L., REN, J., BENNETT, M., ZIA, N., ADAMS, S., WANG, E., ASCENSAO, J., SCHECHTER, G.*, et al.* 2009. Gene and microRNA analysis of neutrophils from patients with polycythemia vera and essential thrombocytosis: down-regulation of micro RNA-1 and -133a. *J Transl Med,* 7**,** 39.

SOMKRUA, R., EICKMAN, E. E., SAOKAEW, S., LOHITNAVY, M. & CHAIYAKUNAPRUK, N. 2011. Association of HLA-B*5801 allele and allopurinol-induced Stevens Johnson syndrome and toxic epidermal necrolysis: a systematic review and meta-analysis. *BMC Med Genet,* 12**,** 118.

SONKOLY, E., STAHLE, M. & PIVARCSI, A. 2008. MicroRNAs: novel regulators in skin inflammation. *Clin Exp Dermatol,* 33**,** 312-5.

SPRAGGS, C. F., BUDDE, L. R., BRILEY, L. P., BING, N., COX, C. J., KING, K. S., WHITTAKER, J. C., MOOSER, V. E., PRESTON, A. J., STEIN, S. H.*, et al.* 2011. HLA-DQA1*02:01 is a major risk factor for lapatinib-induced hepatotoxicity in women with advanced breast cancer. *J Clin Oncol,* 29**,** 667-73.

STANCZYK, J., OSPELT, C., KAROUZAKIS, E., FILER, A., RAZA, K., KOLLING, C., GAY, R., BUCKLEY, C. D., TAK, P. P., GAY, S.*, et al.* 2011. Altered expression of microRNA-203 in rheumatoid arthritis synovial fibroblasts and its role in fibroblast activation. *Arthritis Rheum,* 63**,** 373-81.

STANCZYK, J., PEDRIOLI, D. M., BRENTANO, F., SANCHEZ-PERNAUTE, O., KOLLING, C., GAY, R. E., DETMAR, M., GAY, S. & KYBURZ, D. 2008. Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis. *Arthritis Rheum,* 58**,** 1001-9.

STARKEY LEWIS, P. J., DEAR, J., PLATT, V., SIMPSON, K. J., CRAIG, D. G., ANTOINE, D. J., FRENCH, N. S., DHAUN, N., WEBB, D. J., COSTELLO, E. M.*, et al.* 2011. Circulating microRNAs as potential markers of human drug-induced liver injury. *Hepatology,* 54**,** 1767-1776.

STEIMLE, C., LEHMANN, U., TEMERINAC, S., GOERTTLER, P. S., KREIPE, H., MEINHARDT, G., HEIMPEL, H. & PAHL, H. L. 2007. Biomarker analysis in polycythemia vera under

interferon-alpha treatment: clonality, EEC, PRV-1, and JAK2 V617F. *Ann Hematol,* 86**,** 239-44.

STENZEL, A., LU, T., KOCH, W. A., HAMPE, J., GUENTHER, S. M., DE LA VEGA, F. M., KRAWCZAK, M. & SCHREIBER, S. 2004. Patterns of linkage disequilibrium in the MHC region on human chromosome 6p. *Hum Genet,* 114**,** 377-85.

STERN, J. O., ROBINSON, P. A., LOVE, J., LANES, S., IMPERIALE, M. S. & MAYERS, D. L. 2003. A comprehensive hepatic safety analysis of nevirapine in different populations of HIV infected patients. *J Acquir Immune Defic Syndr,* 34 Suppl 1**,** S21-33.

STOHR, W., BACK, D., DUNN, D., SABIN, C., WINSTON, A., GILSON, R., PILLAY, D., HILL, T., AINSWORTH, J., POZNIAK, A.*, et al.* 2008. Factors influencing efavirenz and nevirapine plasma concentration: effect of ethnicity, weight and co-medication. *Antivir Ther,* 13**,** 675-85.

STRONCEK, D. 2002. Neutrophil alloantigens. *Transfus Med Rev,* 16**,** 67-75.

STRONCEK, D. F. 2007. Neutrophil-specific antigen HNA-2a, NB1 glycoprotein, and CD177. *Curr Opin Hematol,* 14**,** 688-93.

STRONCEK, D. F., SHANKAR, R., LITZ, C. & CLEMENT, L. 1998. The expression of the NB1 antigen on myeloid precursors and neutrophils from children and umbilical cords. *Transfus Med,* 8**,** 119-23.

SUZUKI, Y., INAGI, R., AONO, T., YAMANISHI, K. & SHIOHARA, T. 1998. Human herpesvirus 6 infection as a risk factor for the development of severe drug-induced hypersensitivity syndrome. *Arch Dermatol,* 134**,** 1108-12.

SVENSSON, C. K., COWEN, E. W. & GASPARI, A. A. 2001. Cutaneous drug reactions. *Pharmacol Rev,* 53**,** 357-79.

SZKLARCZYK, D., FRANCESCHINI, A., KUHN, M., SIMONOVIC, M., ROTH, A., MINGUEZ, P., DOERKS, T., STARK, M., MULLER, J., BORK, P.*, et al.* 2011. The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. *Nucleic Acids Res,* 39**,** D561-8.

TAGUCHI, Y. H. & MURAKAMI, Y. 2013. Principal component analysis based feature extraction approach to identify circulating microRNA biomarkers. *PLoS One,* 8**,** e66714.

TAIWO, B. O. 2006. Nevirapine toxicity. *Int J STD AIDS,* 17**,** 364-9; quiz 370.

TAKAHASHI, M., CUATRECASAS, M., BALAGUER, F., HUR, K., TOIYAMA, Y., CASTELLS, A., BOLAND, C. R. & GOEL, A. 2012. The Clinical Significance of MiR-148a as a Predictive Biomarker in Patients with Advanced Colorectal Cancer. *Plos One,* 7.

TANG, J., COSTELLO, C., KEET, I. P., RIVERS, C., LEBLANC, S., KARITA, E., ALLEN, S. & KASLOW, R. A. 1999. HLA class I homozygosity accelerates disease progression in human immunodeficiency virus type 1 infection. *AIDS Res Hum Retroviruses,* 15**,** 317-24.

TANTILLO, C., DING, J., JACOBO-MOLINA, A., NANNI, R. G., BOYER, P. L., HUGHES, S. H., PAUWELS, R., ANDRIES, K., JANSSEN, P. A. & ARNOLD, E. 1994. Locations of anti-AIDS drug binding sites and resistance mutations in the three-dimensional structure of HIV-1 reverse transcriptase. Implications for mechanisms of drug inhibition and resistance. *J Mol Biol,* 243**,** 369-87.

TARANTINO, G., DI MINNO, M. N. & CAPONE, D. 2009. Drug-induced liver injury: is it somehow foreseeable? *World J Gastroenterol,* 15**,** 2817-33.

TASSANEEYAKUL, W., JANTARAROUNGTONG, T., CHEN, P., LIN, P. Y., TIAMKAO, S., KHUNARKORNSIRI, U., CHUCHERD, P., KONYOUNG, P., VANNAPRASAHT, S., CHOONHAKARN, C.*, et al.* 2009. Strong association between HLA-B*5801 and allopurinol-induced Stevens-Johnson syndrome and toxic epidermal necrolysis in a Thai population. *Pharmacogenet Genomics,* 19**,** 704-9.

TASSANEEYAKUL, W., TIAMKAO, S., JANTARAROUNGTONG, T., CHEN, P., LIN, S. Y., CHEN, W. H., KONYOUNG, P., KHUNARKORNSIRI, U., AUVICHAYAPAT, N., PAVAKUL, K.*, et al.* 2010. Association between HLA-B*1502 and carbamazepine-induced severe cutaneous adverse drug reactions in a Thai population. *Epilepsia,* 51**,** 926-30.

TAYLOR, D. D. & GERCEL-TAYLOR, C. 2008. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol Oncol,* 110**,** 13-21.

TEMERINAC, S., KLIPPEL, S., STRUNCK, E., RODER, S., LUBBERT, M., LANGE, W., AZEMAR, M., MEINHARDT, G., SCHAEFER, H. E. & PAHL, H. L. 2000. Cloning of PRV-1, a novel member of the uPAR receptor superfamily, which is overexpressed in polycythemia rubra vera. *Blood,* 95**,** 2569-76.

THE NORDIC COCHRANE CENTRE, Review Manager (RevMan) [Computer Program]. Version 5.2. Copenhagen: The Cochrane Collaboration, 2012.

THIEN, F. C. 2006. 3. Drug hypersensitivity. *Med J Aust,* 185**,** 333-8.

THOMAS, R., APPS, R., QI, Y., GAO, X., MALE, V., O'HUIGIN, C., O'CONNOR, G., GE, D., FELLAY, J., MARTIN, J. N.*, et al.* 2009. HLA-C cell surface expression and control of HIV/AIDS correlate with a variant upstream of HLA-C. *Nat Genet,* 41**,** 1290-4.

TOMANKOVA, T., PETREK, M., GALLO, J. & KRIEGOVA, E. 2011. MicroRNAs: emerging regulators of immune-mediated diseases. *Scand J Immunol*.

TORTI, C., COSTARELLI, S., DE SILVESTRI, A., QUIROS-ROLDAN, E., LAPADULA, G., COLOGNI, G., PARANINFO, G., CASTELNUOVO, F., PUOTI, M., CAROSI, G.*, et al.* 2007. Analysis of severe hepatic events associated with nevirapine-containing regimens: CD4+ T-cell count and gender in hepatitis C seropositive and seronegative patients. *Drug Saf,* 30**,** 1161-9.

TREE STAR INC. 2013. *FlowJo* [Online]. Ashland, Oregon: TreeStar. Available: www.flowjo.com [2013].

TRKOLA, A. 2004. HIV-host interactions: vital to the virus and key to its inhibition. *Curr Opin Microbiol,* 7**,** 407-11.

TSIBRIS, A. M. & HIRSCH, M. S. 2010. Antiretroviral therapy in the clinic. *J Virol,* 84**,** 5458-64.

TSUJIURA, M., ICHIKAWA, D., KOMATSU, S., SHIOZAKI, A., TAKESHITA, H., KOSUGA, T., KONISHI, H., MORIMURA, R., DEGUCHI, K., FUJIWARA, H.*, et al.* 2010. Circulating microRNAs in plasma of patients with gastric cancers. *Br J Cancer,* 102**,** 1174-9.

TURNER, D. 2004. The human leucocyte antigen (HLA) system. *Vox Sang,* 87 Suppl1**,** 87-90.

UETRECHT, J. 2007. Idiosyncratic drug reactions: current understanding. *Annu Rev Pharmacol Toxicol,* 47**,** 513-39.

UETRECHT, J. 2008. Idiosyncratic drug reactions: past, present, and future. *Chem Res Toxicol,* 21**,** 84-92.

UETRECHT, J. & NAISBITT, D. J. 2013. Idiosyncratic adverse drug reactions: current concepts. *Pharmacol Rev,* 65**,** 779-808.

UMAPATHY, S., PAWAR, A., BAJPAI, S., PAZARE, A. R. & GHOSH, K. 2011. HLA involvement in nevirapine-induced dermatological reaction in antiretroviral-treated HIV-1 patients. *J Pharmacol Pharmacother,* 2**,** 114-5.

UNAIDS. 2012a. *Joint United Nations Programme on HIV/AIDS - Malawi* [Online]. Available: http://www.unaids.org/en/regionscountries/countries/malawi/ [Accessed October 2013].

UNAIDS 2012b. UNAIDS Report on the Global AIDS Epidemic.

VELDKAMP, A. I., WEVERLING, G. J., LANGE, J. M., MONTANER, J. S., REISS, P., COOPER, D. A., VELLA, S., HALL, D., BEIJNEN, J. H. & HOETELMANS, R. M. 2001. High exposure to nevirapine in plasma is associated with an improved virological response in HIV-1-infected individuals. *AIDS,* 15**,** 1089-95.

VIDEM, V. & STRAND, E. 2004. Changes in neutrophil surface-receptor expression after stimulation with FMLP, endotoxin, interleukin-8 and activated complement compared to degranulation. *Scand J Immunol,* 59**,** 25-33.

VITEZICA, Z. G., MILPIED, B., LONJOU, C., BOROT, N., LEDGER, T. N., LEFEBVRE, A. & HOVNANIAN, A. 2008. HLA-DRB1*01 associated with cutaneous hypersensitivity induced by nevirapine and efavirenz. *AIDS,* 22**,** 540-1.

VOLBERDING, P. A. & DEEKS, S. G. 2010. Antiretroviral therapy and management of HIV infection. *Lancet,* 376**,** 49-62.

VON VIETINGHOFF, S., TUNNEMANN, G., EULENBERG, C., WELLNER, M., CRISTINA CARDOSO, M., LUFT, F. C. & KETTRITZ, R. 2007. NB1 mediates surface expression of the ANCA antigen proteinase 3 on human neutrophils. *Blood,* 109**,** 4487-93.

WANG, D., CURTIS, A., PAPP, A. C., KOLETAR, S. L. & PARA, M. F. 2012a. Polymorphism in glutamate cysteine ligase catalytic subunit (GCLC) is associated with sulfamethoxazole-induced hypersensitivity in HIV/AIDS patients. *BMC Med Genomics,* 5**,** 32.

WANG, D., PARA, M. F., KOLETAR, S. L. & SADEE, W. 2011a. Human N-acetyltransferase 1 *10 and *11 alleles increase protein expression through distinct mechanisms and associate with sulfamethoxazole-induced hypersensitivity. *Pharmacogenet Genomics,* 21**,** 652-64.

WANG, K., YUAN, Y., CHO, J. H., MCCLARTY, S., BAXTER, D. & GALAS, D. J. 2012b. Comparing the MicroRNA spectrum between serum and plasma. *PLoS One,* 7**,** e41561.

WANG, Q., ZHOU, J. Q., ZHOU, L. M., CHEN, Z. Y., FANG, Z. Y., CHEN, S. D., YANG, L. B., CAI, X. D., DAI, Q. L., HONG, H.*, et al.* 2011b. Association between HLA-B*1502 allele and carbamazepine-induced severe cutaneous adverse reactions in Han people of southern China mainland. *Seizure,* 20**,** 446-8.

WATANABE, I., TOMITA, A., SHIMIZU, M., SUGAWARA, M., YASUMO, H., KOISHI, R., TAKAHASHI, T., MIYOSHI, K., NAKAMURA, K., IZUMI, T.*, et al.* 2003. A study to survey susceptible genetic factors responsible for troglitazone-associated hepatotoxicity in Japanese patients with type 2 diabetes mellitus. *Clin Pharmacol Ther,* 73**,** 435-55.

WATANABE, Y. & KANAI, A. 2011. Systems Biology Reveals MicroRNA-Mediated Gene Regulation. *Front Genet,* 2**,** 29.

WATERS, L. J., MANDALIA, S., GAZZARD, B. & NELSON, M. 2007. Prospective HLA-B*5701 screening and abacavir hypersensitivity: a single centre experience. *AIDS,* 21**,** 2533-4.

WEI, C. Y., LEE, M. T. & CHEN, Y. T. 2012. Pharmacogenomics of adverse drug reactions: implementing personalized medicine. *Hum Mol Genet,* 21**,** R58-65.

WETTER, D. A. & CAMILLERI, M. J. 2010. Clinical, etiologic, and histopathologic features of Stevens-Johnson syndrome during an 8-year period at Mayo Clinic. *Mayo Clin Proc,* 85**,** 131-8.

WHO 1972. International drug monitoring: the role of national centres. *Technical Report Series.* Geneva: World Health Organization.

WHO 2011. Progress report 2011: Globel HIV/AIDS response. Epidemic update and health sector progress towards universal access WHO, UNICEF, UNAIDS.

WILLIAMS, R. T. 1959. *Detoxification Mechanisms: The Metabolism and Detoxification of Drugs, Toxic Substances and Other Organic Compounds*, Chapman and Hall.

WIT, F. W., KESSELRING, A. M., GRAS, L., RICHTER, C., VAN DER ENDE, M. E., BRINKMAN, K., LANGE, J. M., DE WOLF, F. & REISS, P. 2008. Discontinuation of nevirapine because of hypersensitivity reactions in patients with prior treatment experience, compared with treatment-naive patients: the ATHENA cohort study. *Clin Infect Dis,* 46**,** 933-40.

WITTMANN, J. & JÄCK, H.-M. 2010. Serum microRNAs as powerful cancer biomarkers. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer,* 1806**,** 200-207.

WOLFF, J., BRENDEL, C., FINK, L., BOHLE, R. M., KISSEL, K. & BUX, J. 2003. Lack of NB1 GP (CD177/HNA-2a) gene transcription in NB1 GP- neutrophils from NB1 GP-expressing individuals and association of low expression with NB1 gene polymorphisms. *Blood,* 102**,** 731-3.

WOODFIN, A., VOISIN, M. B., IMHOF, B. A., DEJANA, E., ENGELHARDT, B. & NOURSHARGH, S. 2009. Endothelial cell activation leads to neutrophil transmigration as supported by the sequential roles of ICAM-2, JAM-A, and PECAM-1. *Blood,* 113**,** 6246-57.

WU, F., ZIKUSOKA, M., TRINDADE, A., DASSOPOULOS, T., HARRIS, M. L., BAYLESS, T. M., BRANT, S. R., CHAKRAVARTI, S. & KWON, J. H. 2008. MicroRNAs are differentially expressed in ulcerative colitis and alter expression of macrophage inflammatory peptide-2 alpha. *Gastroenterology,* 135**,** 1624-1635 e24.

WU, X. T., HU, F. Y., AN, D. M., YAN, B., JIANG, X., KWAN, P., STEFAN, H. & ZHOU, D. 2010. Association between carbamazepine-induced cutaneous adverse drug reactions and the HLA-B*1502 allele among patients in central China. *Epilepsy Behav,* 19**,** 405-8.

WU, Y., FARRELL, J., PIRMOHAMED, M., PARK, B. K. & NAISBITT, D. J. 2007. Generation and characterization of antigen-specific CD4+, CD8+, and CD4+CD8+ T-cell clones from patients with carbamazepine hypersensitivity. *J Allergy Clin Immunol,* 119**,** 973-81.

WYEN, C., HENDRA, H., VOGEL, M., HOFFMANN, C., KNECHTEN, H., BROCKMEYER, N. H., BOGNER, J. R., ROCKSTROH, J., ESSER, S., JAEGER, H.*, et al.* 2008. Impact of CYP2B6 983T>C polymorphism on non-nucleoside reverse transcriptase inhibitor plasma concentrations in HIV-infected patients. *J Antimicrob Chemother,* 61**,** 914-8.

XU, P., GUO, M. & HAY, B. A. 2004. MicroRNAs and the regulation of cell death. *Trends Genet,* 20**,** 617-24.

XU, Q., JIANG, Y., YIN, Y., LI, Q., HE, J., JING, Y., QI, Y. T., XU, Q., LI, W., LU, B.*, et al.* 2013. A regulatory circuit of miR-148a/152 and DNMT1 in modulating cell transformation and tumor angiogenesis through IGF-IR and IRS1. *J Mol Cell Biol,* 5**,** 3-13.

YAMAMOTO, Y., KOSAKA, N., TANAKA, M., KOIZUMI, F., KANAI, Y., MIZUTANI, T., MURAKAMI, Y., KURODA, M., MIYAJIMA, A., KATO, T.*, et al.* 2009. MicroRNA-500 as a potential diagnostic marker for hepatocellular carcinoma. *Biomarkers,* 14**,** 529-38.

YARCHOAN, R., KLECKER, R. W., WEINHOLD, K. J., MARKHAM, P. D., LYERLY, H. K., DURACK, D. T., GELMANN, E., LEHRMAN, S. N., BLUM, R. M., BARRY, D. W.*, et al.* 1986. Administration of 3'-azido-3'-deoxythymidine, an inhibitor of HTLV-III/LAV replication, to patients with AIDS or AIDS-related complex. *Lancet,* 1**,** 575-80.

YAWALKAR, N., EGLI, F., HARI, Y., NIEVERGELT, H., BRAATHEN, L. R. & PICHLER, W. J. 2000. Infiltration of cytotoxic T cells in drug-induced cutaneous eruptions. *Clin Exp Allergy,* 30**,** 847-55.

YIP, V. L., MARSON, A. G., JORGENSEN, A. L., PIRMOHAMED, M. & ALFIREVIC, A. 2012. HLA genotype and carbamazepine-induced cutaneous adverse drug reactions: a systematic review. *Clin Pharmacol Ther,* 92**,** 757-65.

YOON, V., FRIDKIS-HARELI, M., MUNISAMY, S., LEE, J., ANASTASIADES, D. & STEVCEVA, L. 2010. The GP120 molecule of HIV-1 and its interaction with T cells. *Curr Med Chem,* 17**,** 741-9.

YOUNG, B., SQUIRES, K., PATEL, P., DEJESUS, E., BELLOS, N., BERGER, D., SUTHERLAND-PHILLIPS, D. H., LIAO, Q., SHAEFER, M. & WANNAMAKER, P. 2008. First large, multicenter, open-label study utilizing HLA-B*5701 screening for abacavir hypersensitivity in North America. *AIDS,* 22**,** 1673-5.

YU, J., PENG, H., RUAN, Q., FATIMA, A., GETSIOS, S. & LAVKER, R. M. 2010. MicroRNA-205 promotes keratinocyte migration via the lipid phosphatase SHIP2. *FASEB J,* 24**,** 3950-9.

YU, J., RYAN, D. G., GETSIOS, S., OLIVEIRA-FERNANDES, M., FATIMA, A. & LAVKER, R. M. 2008. MicroRNA-184 antagonizes microRNA-205 to maintain SHIP2 levels in epithelia. *Proc Natl Acad Sci U S A,* 105**,** 19300-5.

YUAN, J., GUO, S., HALL, D., CAMMETT, A. M., JAYADEV, S., DISTEL, M., STORFER, S., HUANG, Z., MOOTSIKAPUN, P., RUXRUNGTHAM, K.*, et al.* 2011. Toxicogenomics of nevirapine-associated cutaneous and hepatic adverse events among populations of African, Asian, and European descent. *AIDS,* 25**,** 1271-80.

YUAN, K., LIAN, Z. R., SUN, B., CLAYTON, M. M., NG, I. O. L. & FEITELSON, M. A. 2012. Role of miR-148a in Hepatitis B Associated Hepatocellular Carcinoma. *Plos One,* 7.

ZHANG, H., LI, Y., HUANG, Q., REN, X., HU, H., SHENG, H. & LAI, M. 2011a. MiR-148a promotes apoptosis by targeting Bcl-2 in colorectal cancer. *Cell Death and Differentiation,* 18**,** 1702-1710.

ZHANG, Y., WANG, J., ZHAO, L. M., PENG, W., SHEN, G. Q., XUE, L., ZHENG, X. X., HE, X. J., GONG, C. Y. & MIAO, L. Y. 2011b. Strong association between HLA-B*1502 and carbamazepine-induced Stevens-Johnson syndrome and toxic epidermal necrolysis in mainland Han Chinese patients. *Eur J Clin Pharmacol,* 67**,** 885-7.

ZHENG, Y., GARDNER, S. E. & CLARKE, M. C. 2011. Cell death, damage-associated molecular patterns, and sterile inflammation in cardiovascular disease. *Arterioscler Thromb Vasc Biol,* 31**,** 2781-6.

ZHOU, X., ZHAO, F., WANG, Z. N., SONG, Y. X., CHANG, H., CHIANG, Y. P. & XU, H. M. 2012. Altered expression of miR-152 and miR-148a in ovarian cancer is related to cell proliferation. *Oncology Reports,* 27**,** 447-454.

ZHU, W., QIN, W., ATASOY, U. & SAUTER, E. R. 2009. Circulating microRNAs in breast cancer and healthy subjects. *BMC Res Notes,* 2**,** 89.

ZIMMERMAN, H. J. 1999. *Hepatotoxicity : the adverse effects of drugs and other chemicals on the liver,* Philadelphia, Lippincott Williams & Wilkins.

ZUCMAN, D., TRUCHIS, P., MAJERHOLC, C., STEGMAN, S. & CAILLAT-ZUCMAN, S. 2007. Prospective screening for human leukocyte antigen-B*5701 avoids abacavir hypersensitivity reaction in the ethnically mixed French HIV population. *J Acquir Immune Defic Syndr,* 45**,** 1-3.

ZWIERS, A., KRAAL, L., VAN DE POUW KRAAN, T. C., WURDINGER, T., BOUMA, G. & KRAAL, G. 2012. Cutting edge: a variant of the IL-23R gene associated with inflammatory bowel disease induces loss of microRNA regulation and enhanced protein production. *J Immunol,* 188**,** 1573-7.